The Detection and Molecular Characterisation of Orchid Fleck Virus.

by

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Declaration

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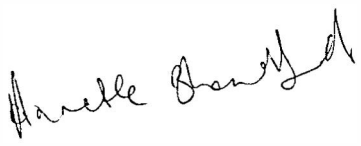

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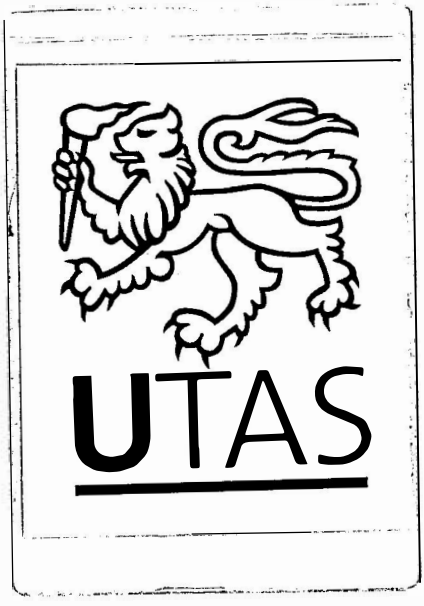

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3. Acknowledgments

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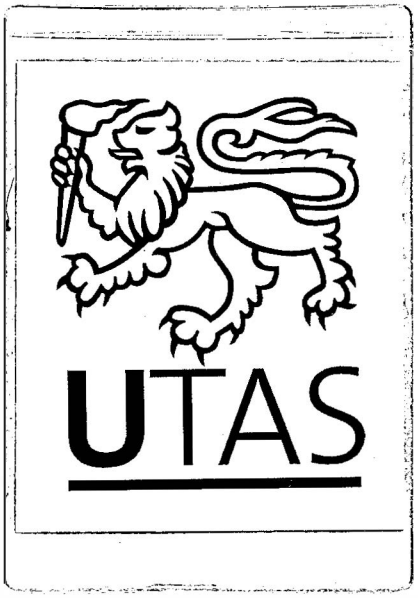


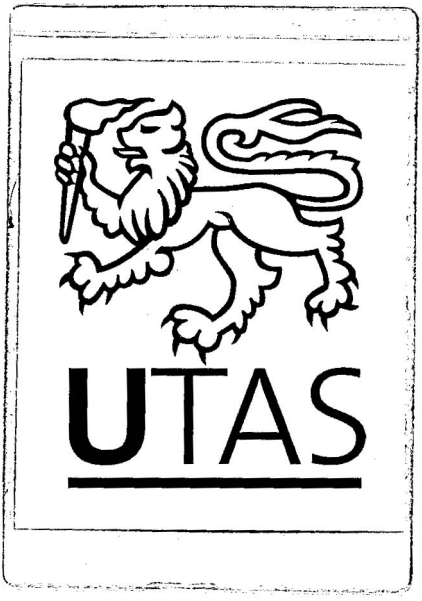
4. Abstract

Orchid fleck virus (OFV) is one of three commonly found viruses infecting orchids. Infected plants have reduced flower quality and often unsightly leaf markings and are unsaleable. Effective disease control relies on routine detection of the virus, but foliar symptoms can vary markedly and are often not reliable for diagnosis. Current laboratory tests use examination of leaf sap by electron microscopy. This is time consuming and costly. The preferred immunological tests are not available as OFV has an unstable virion and attempts to purify virus particles for antibody production have failed. However, direct isolation of viral nucleic acid from infected plants may enable development of alternative detection systems.

In this project OFV was characterised by mechanical inoculation to alternate hosts. Sap inoculation was found to be difficult and affected by glasshouse temperatures, however, was successful at temperatures significantly lower than previously reported. The host range of a Tasmanian isolate of OFV was found to be different to that previously reported for isolates of OFV from Japan. Attempts to purify OFV and clone the viral RNA were unsuccessful, however primers specific to OFV were obtained.

OFV was detected using RT-PCR with a primer complementary to a region of its nucleoprotein gene together with a polydT/SP6 primer complementary to the 3' terminus of the genomic segment. The resulting DNA fragments were 0.8kb long and their sequences were determined directly. The sequences of DNA fragments obtained from 33 OFV isolates from Australia, Brazil, Germany and South Africa were shown to be closely related (<2.5% difference), but a single German isolate was clearly a distinct strain and the sequence of the targeted region of its nucleoprotein gene differed from that of the others by about 16%. Failure of RT-PCR using a second primer set complimentary to part of the phosphoprotein gene of the Japanese OFV isolate with all OFV isolates tested suggests that the Japanese isolate may represent a third distinct strain of OFV or a different virus. A search of the international nucleotide database with the OFV sequences showed them to be related, but distantly, to regions of the genomes of three plant rhabdoviruses.

Isolates of coffee ringspot virus (CoRSV), citrus leprosis virus (CiLV), a common violet (Viola sp.), schefflera, hibiscus, ivy and ligustrum leaves showing ringspot symptoms and containing small bacilliform virus particles were tested using RT-PCR and the OFV specific primers. A single product of 800bp was amplified from one isolate of coffee ringspot virus and the violet sample using the primer complementary to a region of the OFV nucleoprotein gene together with a polydT/SP6 primer. The DNA products were shown to be identical to OFV when sequenced. No other sample gave an amplified product. These results suggest citrus leprosis disease, ligustrum ringspot and the ringspots on schefflera, hibiscus and ivy are caused by viruses different to OFV. However, this study was completed with a limited number of samples and the results are not conclusive, the relationship between these viruses should be further investigated.



5. Literature Review - The Plant Rhabdovirus Group

5.1 Introduction

The Rhabdoviridae represent one of only two virus families that include members capable of infecting either vertebrates or plants (Matthews, 1982). Members of both groups are transmitted by insects, in which they are propagative. Rhabdoviruses have complex bacilliform or bullet-shaped virions composed of RNA, protein, carbohydrate and lipid and are easily recognised in electron-microscopic examination of plant sap (Jackson *et al.*, 1987). The importance of rhabdoviruses as disease agents and their potential danger to human and livestock health is well documented (Brown and Crick, 1979). However, rhabdoviruses also cause serious diseases of plants, resulting in substantial crop losses.

Rhabdoviruses are found in tropical, subtropical and temperate regions and are known to infect most major crop plants (Jackson *et al.*, 1987). Particles similar to rhabdoviruses have been observed in several species of plants showing disease symptoms, but the viral nature of the particles has not been supported by adequate transmission experiments (Francki, 1973). The most important factor limiting studies of plant rhabdoviruses is the difficulty of developing simple and reproducible purification protocols suitable for recovery of adequate amounts of virus of sufficient purity for biochemical analysis (Jackson *et al.*, 1987).

5.2 Biological properties

5.2.1 Geographic distribution and host range

Rhabdoviruses have been reported from most parts of the world including tropical, subtropical and temperate regions (Peters, 1981). The majority have restricted distributions and this probably reflects the distributions of their vectors. Rhabdoviruses infect most major crop plants, and in many instances, serious disease outbreaks have been reported (Jackson *et al.* 1987). The natural host range of individual members is narrow and often limited to one or a few plant species (Peters, 1981). However, eggplant mottled dwarf virus has been detected in eggplant, tomato,

hibiscus, honeysuckle, potato and weed hosts in several locations around the Mediterranean basin (Danesh and Lockhart, 1989). Rhabdoviruses have been reported in grasses from all parts of the world, but their relationships have not been studied (Peters, 1981).

5.2.2 Ecology and Pathology

Rhabdoviruses cause various symptoms in monocotyledonous and dicotyledonous plants, similar to those caused by infection with viruses belonging to numerous other groups (Jackson *et al.*, 1987). Symptoms therefore have no diagnostic value because of their highly variably nature, ranging from patterns of chromatic discolouration of the foliage to flower colour break, localised to extensive necrosis, stunting, and a reduction in the yield of fruits or seeds and latent infections (Jackson *et al.*, 1987; Peters, 1994). Among dicotyledons, potato yellow dwarf (PYDV) and lettuce necrotic yellows (LNYV) viruses cause diseases of economic importance, whereas eggplant mottled dwarf (EMDV) and lucerne enation (LEV) viruses are potentially destructive pathogens owing to the severity of the symptoms they elicit. In monocotyledonous plants, at least six of the eight known cereal rhabdoviruses are responsible for severe field diseases. Maize mosaic virus has been reported as causing yield losses of 100% in maize crops. Rice transitory yellowing virus causes serious decreases in yield in rice in the central and southern region of Taiwan, where two rice crops are grown in the one season (Shikata, 1972).

5.2.3 Transmission

The aetiology and epidemiology of most plant infecting rhabdoviruses are poorly understood. However, plant rhabdoviruses are believed to be transmitted by plant-sucking arthropods in a circulative and propagative manner. Of the 25 members listed by Peters (1981), whose vectors are known, all except two are transmitted by species of the order *Hemiptera.* Most rhabdoviruses are transmitted by aphids, leafhoppers or planthoppers (Peters, 1994). Beet leaf curl virus is transmitted by the lacebug *Piesma quadratum* and coffee ringspot virus and several other tentative members of the

rhabdovirus family are transmitted by *Brevipalpus* mites (Peters, 1981; Rossetti *et al.*, 1998). The virus-vector interaction is highly specific. Studies on the vectors of plant rhabdoviruses have shown the viruses multiply in both plants and insects (Jackson *et al.*, 1987). None of the viruses appears to adversely affect the insects, which suggests a long evolutionary association between them (Jackson *et al.*, 1987). A small proportion of the plant rhabdoviruses can be mechanically transmitted to a restricted range of herbaceous plants (Peters, 1981).

5.3 Virion properties

5.3.1 Morphology

Particles of rhabdoviruses infecting vertebrates and invertebrates usually appear bullet-shaped or cone-shaped (Wunner *et al.*, 1995). The particles of rhabdoviruses infecting plants often appear bacilliform when fixed prior to negative staining, whilst in unfixed preparations they can appear bullet-shaped or pleomorphic (Wunner *et al.*, 1995). The two types of particle are morphologically distinct, bacilliform with both ends rounded and bullet-shaped with one rounded and one planar end. Purified preparations of plant rhabdoviruses with a bacilliform appearance *in situ* often contain a high number of bullet-shaped particles, indicating that either the bacilliform shape is an unstable form (Peters, 1994) or that only the mature forms are bacilliform in shape (Knudson, 1973; Francki, 1973). RTYV represents an exception, for its particles are reported to be primarily of the bullet type, in both preparations (Shikata, 1972).

Plant-infecting rhabdoviruses vary more in size than animal rhabdoviruses (Peters, 1994). Plant rhabdovirus virions vary in length from 100 to 430nm and in diameter from 45 to 100nm (Wunner *et al.*, 1995). Defective particles are proportionately shorter. Abnormally long, double-length particles and tandem formations are also observed. Most possess a membrane envelope which surrounds the nucleocapsid, but some putative members of the group lack envelopes (Wunner *et al.*, 1995). Projections, termed peplomers (5-10nm long) protrude from the outer surface of virions (Wunner *et al.*, 1995). They consist of trimers of the virus glycoprotein. A

honeycomb pattern of peplomers is observed on the surface of some viruses (Wunner *et al.*, 1995). The nucleocapsid, about 30-70nm in diameter, forms an internal, coiled helix with a hemispherical and a blunt end. It is formed by a helically wound nucleoprotein strand composed of a single-stranded RNA genome and a nucleocapsid protein (N) (Peters, 1981). It can be seen as cross-striations in negatively stained and thin-sectioned virus particles. The nucleocapsid contains transcriptase activity and is infectious (Wunner and Peters, 1991). Uncoiled it is filamentous, about 700nm long and 20nm in diameter (Wunner *et al.*, 1995).

5.3.2 Genome properties

Rhabdovirus particles contain a single molecule of linear, single-stranded, negative-sense RNA (MW 4.2-4.6 x 10^6), ie., it is complementary to its messenger RNA (mRNA) species produced in cells. The RNA consists of 11-15 kilobases and represents about 1-2% of particle weight (Wunner *et al.*, 1995). The RNA has 5' terminal triphosphate and is not polyadenylated. The ends have inverted complementary sequences. The mRNA of Sonchus yellow net virus (SYNV) is polyadenylated at the 3' end (Rezaian *et al.*, 1983). The genome RNA by itself is not infective. The genome of orchid fleck virus (OFV) is single stranded RNA, but unlike rhabdoviruses, is bipartite with each component approximately 6kb in length and polyadenylated at the 3' terminus (Kondo *et al.*, 1998). Relative to animal rhabdoviruses, plant rhabdoviruses often have larger genomes, as they possess an extra gene which encodes a protein required to transport the virus between cells (Peters, 1994). The genome of SYNV contains more than 14,000 nucleotides (Rezaian *et al.*, 1983; Dietzgen *et al.*, 1989), which is 1.18 times longer than that of the type member vesicular stomatitis virus (VSV) (11,000 nucleotides) (Peters, 1994). The nucleocapsid volumes and size of the plant rhabdovirus genomes do not support the belief of some virologists that plant rhabdoviruses are formed by two bullet-shaped nucleocapsids attached by blunt end aggregation (Peters, 1994).

5.3.3 Proteins, lipids and carbohydrates

The best characterized plant rhabdoviruses are SYNV, LNYV, and potato yellow dwarf virus (PYDV) (Jackson *et al.*, 1987). These viruses have been estimated to contain about 70% protein, 20-25% lipid and 2-3% RNA (Jackson *et al.*, 1987). The distribution of lipids in PYDV and wheat striate (American) mosaic virus are also similar to that in SYNV. In all three cases, fatty acids and sterols predominate, while the proportion of triglycerides is low (Jackson *et al.*, 1987). The virions also contain carbohydrate. The carbohydrates are present as N-linked glycan chains on G protein and as glycolipids (Wunner *et al.*, 1995). Virions are composed of about 3% carbohydrate by weight (Wunner *et al.*, 1995).

The polypeptides of plant rhabdoviruses differ considerably in their size, clearly illustrated by the comparison of the relative sizes of the proteins of SYNV, PYDV and LNYV (Table 1). Particles of the plant rhabdoviruses contain at least four structural proteins. All have a 'nucleocapsid protein' (N) which has a MW 55-60 x 10^3 and a 'glycoprotein' (G) which has a MW 71-93 x 10^3 (Wunner and Peters, 1991). Protein G forms a hexagonal array over the outside of the membrane (Peters, 1981). In addition, plant rhabdoviruses can be grouped according to their protein composition. Viruses of the cytorhabdovirus group have one matrix (M) protein (MW 18-25 x 10^3) and a 'large' protein (L) (MW 145 x 10^3), is detected in some members. Viruses of the nucleorhabdovirus group possess two M proteins (MW 27-44 x 10^3 and 21-39 x 10^3) (Wunner and Peters, 1991).

Table 1 Comparison of the protein species of LNYV, PYDV and SYNV.

Virus	Viral proteins (mol. wt. x 10^3)						References
	L	G	N	P	M1	M2	
Lettuce necrotic yellows virus (LNYV)	170	71	56	38	19	-	Dale and Peters (1981)
Potato yellow dwarf virus (PYDV)	+	78	56	-	33	22	Knudson (1973)
Sonchus yellow net virus (SYNV)	125-150	77	64	-	45	39	Jackson and Christie (1977)

(-) Protein was not detected; (+) protein was present, but molecular weight was not estimated.

An L protein associated with isolated nucleocapsid preparations was detected in LNYV (Dale and Peters, 1981). Comparisons with sowthistle yellow vein virus (SYVV), SYNV and EMDV failed to reveal the presence of an L protein in nucleocapsid preparations, but showed that high-molecular weight proteins are present in the soluble fraction after dissociation of the viral envelope with nonionic detergent (Dale and Peters, 1981; Jackson, 1978; Ziemiecki and Peters, 1976a). Plants infected with SYNV were shown to contain a 6600-nucleotide virus-specific transcript (Rezaian *et al.*, 1983). This putative mRNA has sufficient coding capacity to encode an L protein, suggesting an L protein could have a functional role in replication (Rezaian *et al.*, 1983). The presence of transcriptase activity in virions of LNYV and broccoli necrotic yellows virus also implies the proteins of plant and animal rhabdoviruses have similar functional activities (Toriyama and Peters, 1981).

A G protein has been identified in all plant rhabdoviruses tested (Jackson *et al.*, 1987). The G proteins of LNYV (Francki and Randles, 1975), SYVV (Ziemiecki and Peters, 1976b), SYNV (Jackson, 1978) and PYDV (Adam and Hsu, 1984) have molecular weights varying from 70,000 to 90,000 and stain positively for carbohydrate. The G protein is thought to be part of the viral envelope and appears to be exposed on the surface of virions (Jackson *et al.*, 1987).

All the plant rhabdoviruses that have been studied have an N protein that is tightly complexed with the viral RNA (Francki and Randles, 1975; Ziemiecki and Peters, 1976b; Jackson, 1978; Dale and Peters, 1981). Dissociation of the virus with nonionic detergents releases a core particle that sediments at 200-250 S (Jackson, 1978). With SYNV and LNYV this particle is less infectious than intact virus, but the nucleocapsids retain some infectivity (Jackson, 1978, Randles and Francki, 1972).

A phosphorylated protein with the properties of the P protein has not been unequivocally identified in any plant rhabdovirus (Jackson *et al.*, 1987). However, a protein thought to be a NS protein, is associated with purified nucleocapsids of LNYV (Dale and Peters,

1981). LNYV, BNYV, sonchus virus, maize mosaic virus and tomato vein-yellowing virus (TVYV) are also thought to have a P protein (Dale and Peters, 1981; Falk and Tsai, 1983; El Maataoui *et al.*, 1985).

There is no structural data available about the M proteins of any plant rhabdovirus that allows the proteins that correspond to the P and M proteins of VSV to be distinguished (Jackson *et al.*, 1987). Additional structural information is needed before the P and M functions can be assigned to the proteins of any of the plant rhabdoviruses (Jackson *et al.*, 1987).

5.3.4 Genome organisation

The complete genomes of RTYV (NCBI, AB011257) and SYNV (NCBI, L32603) have been sequenced. The genome of LNYV (NCBI, L24365, L24364, L30103) and orchid fleck virus (OFV) (Kondo *et al.* 1998), previously a putative member of the rhabdovirus family, have been partially sequenced. The genome organisation for LNYV has been described. The genome of OFV is single stranded RNA, but unlike rhabdoviruses, is bipartite with each component approximately 6kb in length and polyadenylated at the 3' terminus (Kondo *et al.*, 1998).

Plant rhabdovirus genomes code for at least 5 ORFs in the negative-sense genome, and with some viruses, additional genes are interposed (Wunner *et al.*, 1995). The genome of RTYV (nucleorhabdovirus) encodes seven genes (l-N-2-3-M-G-6-L-t) (Fang *et al.*, 1998). The genome of SYNV (nucleorhabdovirus) consists of 6 ORFs (3'-N-M2-sc4-M1-G-L-5') separated by dinucleotide GG spacers lying within a common "gene junction" consensus sequence (AUUCUUUUUGGUUGG) with some relatedness to the gene junction regions of VSV and rabies virus (Heaton *et al.*, 1989). Transcription of a rhabdovirus genome is preceded by the synthesis of a leader RNA. The leaders carry functions that regulate transcription and replication (Wunner *et al.*, 1995). The leader of SYNV differs considerably in length from that of VSV and rabies virus (Heaton *et al.*, 1989).

5.3.5 Physicochemical and Physical Properties

The most important factor limiting studies of plant rhabdoviruses is the difficulty of devising simple and reproducible purification protocols for recovery of adequate amounts of virus of sufficient purity for biochemical analysis (Jackson *et al.*, 1987). Rhabdovirus particles are structurally complex and very unstable both in crude extracts of plants or insects and in clarified plant sap analysis (Jackson *et al.*, 1987). Their thermal inactivation point is approximately 50°C and the longevity *in vitro* at room temperature is only a few hours (Peters, 1981). They do not withstand treatment with organic solvents and need to be stabilised in various ways during extraction and purification to retain infectivity. The concentration of rhabdoviruses in infected plants is lower than that of many other plant viruses, generally between 1 to 10mg/l (Peters, 1981; Jackson *et al.*, 1987). Virus buoyant density in CsCl is 1.19-1.20 g/cm^3, and in sucrose it is 1.17-1.19 g/cm^3 (Wunner *et al.* 1995).

5.3.6 Antigenic properties

Plant rhabdoviruses are generally poor immunogens, but polyclonal antisera to several viruses have been prepared (Wunner and Peters, 1991). Some have been shown to contain antibodies to all the virus structural proteins (Wunner and Peters, 1991). G protein is involved in virus neutralization and defines serotype. N protein is a cross-reacting, complement-fixing (CF) antigen (Wunner *et al.*, 1995).

Serology has been used to assess relationships of plant rhabdoviruses. Several of the well characterized viruses have been shown to be antigenically related, however, no serological relationships between members of either subgroup have been established (Peters, 1994). Studies made so far show that viruses transmitted by plant-hoppers between grasses are closely or distinctly related whereas those transmitted by leaf-hoppers

are serologically completely distinct (Peters, 1994). TVYV was shown to be an isolate of EMDV using serological techniques (Adam *et al.*, 1987), however, TVYV was clearly distinguished from PYDV by serology (El Maataoui *et al.*, 1985). Although the relationships between the different plant rhabdoviruses have to be elucidated in more detail, the limited cases in which some relationships were found strengthen the theory that plant rhabdoviruses form a group of many different species (Peters, 1994). Features such as nucleotide sequence homology between the different viruses and genome organization have not been used to study their taxonomic relations. Serological relationships between rhabdoviruses infecting animals and plants have not been reported (Peters, 1994).

5.3.7 Ultrastructure and Replication

Although most animal rhabdoviruses replicate and assemble in the cytoplasm, plant rhabdoviruses differ markedly in their morphogenesis and site of accumulation (Jackson *et al.*, 1987). Electron microscopy has permitted detailed ultrastructural studies of the cytopathology of infected plants. These studies show that plant rhabdoviruses can be divided into at least four groups depending on the site of nucleocapsid formation and assembly of viral particles, and on the cytopathogenic structures encountered in the infected cells (Peters, 1994).

A large group, including SYNV, PYDV, SYVV and EMDV mature in association with the inner nuclear membrane and accumulate in the perinuclear spaces. Extensive aggregation in the perinuclear spaces can lead to the formation of invaginations filled with viral particles in the cytoplasm and or nucleus (Peters, 1994; Jackson *et al.*, 1987). In the case of SYNV, virions often accumulate in disordered arrays around the nucleus (Jackson *et al.*, 1987). A second group of viruses, including LNYV, maize sterile streak virus and broccoli necrotic yellows virus, appear to mature in association with the endoplasmic reticulum and accumulate in vesicles of the endoplasmic reticulum (Peters, 1994; Jackson *et al.*, 1987). A third group of viruses, of which barley yellow striated mosaic virus (BYSMV) and northern cereal mosaic virus are examples, mature in

association with membrane-bound granular structures, called viroplasms (Peters, 1994; Jackson *et al.*, 1987). The particles accumulate in the vacuolelike spaces after budding from the membranes associated with the viroplasms. A fourth group, represented by coffee ringspot virus, accumulate in the nucleus and their nucleocapsids are arranged as spokes in wheel-like structures surrounded by membranes (Peters, 1994). Several rhabdoviruses vary slightly from the patterns of assembly and accumulation described. Virions of these viruses appear to be assembled in or near the nucleus, but virions may also be found in the cytoplasm close to the cytoplasmic membranes. This type of morphogenesis has been extensively studied with wheat striate American mosaic virus (Jackson *et al.*, 1987).

Infection of cowpea protoplasts with SYNV (van Beek *et al.*, 1985a) and festuca leaf streak virus (FLSV) (van Beek et al., 1985b) enabled the analysis of replication events occurring during infection with plant rhabdoviruses. Infectivity of SYNV was detected in extracts of cowpea protoplasts 11-12 hours after inoculation (van Beek *et al.* 1985a),. The infectivity increased until about 30 hours after inoculation and subsequently began to decline (van Beek *et al.*, 1985a). Numerous rhabdovirus particles were present in the perinuclear space and in the cytoplasm of infected protoplasts at 67 hours after inoculation (van Beek *et al.*, 1985a). Replication of FLSV appeared to be slower than that of SYNV, because virus particles did not appear in the protoplasts until 26 hours after inoculation (van Beek et al., 1985b). However, at that time, small viroplasms were observed in the cytoplasm and particles at various stages of morphogenesis were also seen budding from cytoplasmic membranes (van Beek *et al.*, 1985b). In contrasts to cells infected with SYNV, particles of FLSV never became abundant in the cytoplasm and no ultrastructural changes were observed in the nucleus (van Beek *et al.*, 1985b). These results show that FLSV, which has a monocotyledonous hosts, is capable of replicating in protoplasts from dicotyledonous plants. The site of morphogenesis of FLSV differs markedly from that of SYNV. FLSV also appears to replicate inefficiently in the cowpea protoplasts, because the numbers of virus particles were much lower than those observed

in protoplasts infected with SYNV and were also lower than the concentration observed in cells of the native grass host (van Beek et al., 1985b).

Studies by Jones and Jackson (1990) showed transcription of SYNV was initiated soon after infection of tobacco protoplasts as small amounts of SYNV-specific mRNA transcripts were detected within 2 hours after infection and these continued to increase in abundance until at least 24 hours after infection (Jones and Jackson, 1990). mRNA transcripts were reported to reach their highest concentration at 24 hours postinoculation, before appreciable amounts of genomic length RNAs began to accumulate at 36 hours postinoculation. This implies that SYNV mRNA transcription and replication of viral genomic RNA are temporally regulated (Jones and Jackson, 1990). During this time, the four major structural proteins of SYNV accumulated rapidly and replication of genomic RNA was not apparent until significant amounts of structural proteins accumulated. As the viral proteins reached their maximum amounts, the mRNA concentrations declined, as would be expected if transcription, translation, and replication events were coordinately regulated. These results suggest the regulation of SYNV replication may be similar to that of animal rhabdoviruses (Jones and Jackson, 1990).

Defective-interfering (DI) particles are common in animal rhabdovirus preparations maintained by repeated transfer at high multiplicity of infection (Jackson *et al.*, 1987). The genomes of the DI particles lack part of the complete genome and are dependent on the wild-type virus for replication. The DI particles have a distinct replicative advantage over the complete genome and accumulate at the expense of wild-type virus. Only two cases of putative DI particles have been reported in plant rhabdoviruses (Peters, 1994). Repeated transfer of PYDV at high inoculum concentrations, under conditions in which symptoms developed rapidly in tobacco, resulted in a decreased recovery of the virus by purification (Adam *et al.*, 1983). Particles were found which sedimented at a lower rate and had a lower density than the normal particles. When this variant form was mixed with normal particles, the infectivity of the normal particles was significantly reduced, suggesting that the variant fraction contained DI particles (Adam *et al.*, 1983).

Defective SYNV particles were reported in *Nicotiana edwarsonii* plants inoculated with sap extracted from chronically infected calyx tissue (Ismail and Milner, 1988). The infected plants exhibited a chlorotic mottling, instead of the normal vein-clearing symptoms. Shorter particles were purified from the inoculated plants and were not infectious (Ismail and Milner, 1988). Plants inoculated with a mixture of these short and normal particles developed mottling symptoms and yielded predominantly short particles. The RNA of these short particles was 77% of the size of the standard virus (Ismail and Milner, 1988).

5.4 Taxonomic structure of the family

Around 80 plant-infecting rhabdoviruses are currently recognised (Peters, 1994). The International Committee on Taxonomy of Viruses distinguished two subgroups of plant rhabdoviruses, plus additional unassigned viruses. The viruses are primarily distinguished on the basis of the sites of virus maturation, the cytoplasm: *Cytorhabdovirus*; and the perinuclear space: *Nucleorhabdovirus* (Wunner *et al.*, 1995). Exceptions exist and the significance of this property is not known. The interrelationships of the different plant viruses within or between the two genera or with the unassigned plant viruses have yet to be established at the genetic level. In addition, several papers have been published which report the finding of rhabdovirus-like particles by electron microscopy in thin sections of plant cells or leaf dip preparations without providing appropriate virus transmission or other data implicating their relationship to other rhabdoviruses (Jackson *et al.*, 1987).

Taxonomic structure of the family

Family Rhabdoviridae

Animal-infecting rhabdoviruses

Genus *Vesiculovirus*

Genus *Lyssaavirus*

Genus *Ephemerovirus*

Genus *Novirhabdovirus*

Plant-infecting rhabdoviruses

Genus *Cytorhabdovirus*

Genus *Nucleorhabdovirus*

5.4.1 **Genus** Cytorhabdovirus

Type Species lettuce necrotic yellows virus

Cytorhabdoviruses replicate in the cytoplasm of infected cells in association with masses of thread-like structures (viroplasms) (Wunner *et al.*, 1995). Virus morphogenesis occurs in association with vesicles of the endoplasmic reticulum (Wunner *et al.*, 1995). A nuclear phase has been suggested in the replication of some cytorhabdoviruses. Evidence of the nuclear involvement in the replication of others is lacking (Wunner *et al.*, 1995). Their particles contain one protein with low molecular weight, denoted M, and possess a transcriptase activity that can readily be detected *in vitro* (Peters, 1994).

Table 2 Species in the Genus Cytorhabdovirus

Virus and CMI/AAB description No. ( )	Vector	Particle dimensions (nm)
barley yellow striate mosaic virus (BYSMV) (312)	leafhopper	45 x 330
broccoli necrotic yellows virus (BNYV) (85)	aphid	64 x 297
Festuca leaf streak virus (FLSV)	unknown	61 x 330
lettuce necrotic yellows virus (LNYV) (26, 243)	aphid	52 x 360
Northern cereal mosaic (NCMV) (322)	leafhopper	60 x 300/350
Sonchus virus (SV)	unknown	50/70 x 250/300
strawberry crinkle virus (SCV) (163)	aphid	69 x 190/380
wheat American striate mosaic (WASMV) (99)	leafhopper	75 x 250

5.4.2 **Genus** Nucleorhabdovirus

Type Species: potato yellow dwarf virus

Nucleorhabdoviruses multiply in the nucleus of plants forming large granular inclusions that are thought to be sites of virus replication (Wunner *et al.*, 1995). Viral proteins are synthesized from discrete polyadenylated nRNAs and accumulate in the nucleus. Virus morphogenesis occurs at the inner nuclear envelope and enveloped virus particles accumulate in the perinuclear space (Wunner *et al.*, 1995). Their particles possess two low molecular weight proteins, often called M1 and M2, but are presumably matrix (M) and non-structural (NS) proteins, and have low *in vitro* transcriptase activity (Peters, 1994).

Table 3 Species in the Genus Nucleorhabdovirus

Virus and CMI/AAB description No. ( )	Vector	Particle dimensions (nm)
Datura yellow vein virus (DYVV)	unknown	77 x 166
eggplant mottled dwarf virus (EMDV) (115) (Pittosporum vein yellowing virus (PVYV)) (tomato vein yellowing virus (TVYV))	unknown	66 x 220
maize mosaic virus (MMV) (94)	leafhopper	48 x 240
potato yellow dwarf virus (PYDV) (35)	leafhopper	75 x 380
rice transitory yellowing virus (RTYV) (100) (rice yellow stunt virus)	leafhopper	93 x 325
sonchus yellow net virus (SYNV) (205)	aphid	94 x 248
sowthistle yellow vein virus (SYVV) (62)	aphid	95 x 220

Probable members of Plant Rhabdovirus group. Not officially grouped, listed according to type of vector. Transmitted experimentally but not characterised physico-chemically.

Table 4 Aphid transmitted viruses

Virus and CMI/AAB description No. ()	*In vivo* site of assembly	particle dimensions (nm)
carrot latent virus (CaLV)	Nuc.	70 x 220
Lucerne enation virus (LuEV)	Nuc.	82-89 x 250
Parsley rhabdovirus (PRV)	Cyt.	87 x 214
Raspberry vein chlorosis virus (RVCV) (174)	Cyt.	65 x 430

Table 5 Leafbopper- or planthopper-transmitted viruses

Virus and CMI/AAB description No. ()	*In vivo* site of assembly	Particle dimensions (nm)
Cereal chlorotic mottle virus (CeCMV) (251)	Nuc.	63 x 230
Colocasia bobone disease virus (CBDV)	Nuc.	65 x 380-335
Cynodon chlorotic streak virus (CCSV)	Nuc.	72 x 240
Digitaria striate virus (DSV)	Cyt.	55 x 280
Finger millet mosaic virus (FMMMV)	Nuc.	80 x 285
maize sterile stunt virus (MSSV)	Cyt.	45 x 255
oat striate mosaic virus (OSMV)	Nuc.-Cyt.	100 x 400
papaya apical necrosis virus		
Sorghum stunt moaic virus (SSMV)	Nuc.	68 x 220
Shiraz maize rhabdovirus (SMRV)	unknown	70-85 x 150-250
wheat chlorotic streak virus (WCSV)	Cyt.	55 x 355
Wheat rosette stunt virus (WRSV)	Cyt.	40-54 x 320-400
winter wheat (Russian) mosaic virus (WWMV)	Cyt.	60 x 260

Table 6 Lacebug-transmitted virus

Virus and CMI/AAB description No. ()	*In vivo* site of assembly	Particle dimensions (nm)
Beet leafcurl virus (BLCV) (268)	Nuc.	80 x 225-350

Table 7 Mite-transmitted viruses

Virus	*In vivo* site of assembly	particle dimensions (nm)
citrus leprosis virus (CLV)	Nuc.	50-55 x 120-130
coffee ringspot virus (CRV)	Nuc.	59-76 x 178-224

orchid fleck virus (OFV)	Nuc.	32-35 x 100-140

Table 8 Viruses with no known vector

Virus	*In vivo* site of assembly	Particle dimensions (nm)
Cow parsnip mosaic virus (CoPMV)	Nuc.	90 x 265
Cynara virus (CV)	Cyt.	75 x 260
Gomphrena virus (GV)	Nuc.	75 x 230-250
Ivy vein-clearing virus (IVCV)	Nuc.	55 x 325
laburnum yellow vein virus (LYVV)	Nuc.	89 x 245
Moroccan wheat rhabdovirus (MWRV)	unknown	50-60 x 220-240
Melilotus latent virus (MeLV)	Nuc.	80 x 300-350
Pelargonium vein-clearing virus (PLVCV)	Nuc.	70 x 250
Pisum virus (PV)	Cyt.	45 x 240
Raphanus virus (RV)	Cyt.	50-70 x 250-350

Other possible members:

Atropa belladonna virus (AtBV)

Callistephus chinensis chlorosis virus (CCCV)

carnation bacilliform virus (CBV)

cassava symptomless virus (CasSV)

chrysanthemum frutescens virus (CFV)

chrysanthemum vein chlorosis virus (CVCV)

clover enation virus (CloEV)

coriander feathery red vein virus

Euonymus fasciation virus (EFV)

gerbera symptomless virus (GrbSV)

Holcus lanatus yellowing virus (HLYV)

Iris germanica leaf stripe virus (IGLSV)

Laelia red leafspot virus (LRLSV)

Launea arborescens stunt virus (LASV)

lemon scented thyme leaf chlorosis virus (LSTCV)

Lolium ryegrass virus (LoRV)

lotus stem necrosis (LoSNV)

lucerne enation virus (LEV)

lupin yellow vein virus (LYVV)

Malva silvestris virus (MaSV)

Melilotus latent virus (MeLV)

melon variegation virus (MVV)

parsley latent virus (PLV)

pigeon pea proliferation virus (PPPV)

pineapple chlorotic leaf streak virus (PCLSV)

plantain mottle virus (PlMV)

Ranunculus repens symptomless virus (RaRSV)

red clover mosaic virus (RClMV)

Sainpaulia leaf necrosis virus (SLNV)

Sambucus vein clearing virus (SVCV)

Sarracenia purpurea virus (SPV)

Sorghum virus (SV)

Sorghum stunt virus (SSV)

soursop yellow blotch virus (SYBV)

Thunbergia alata rhabdovirus (TaRV)

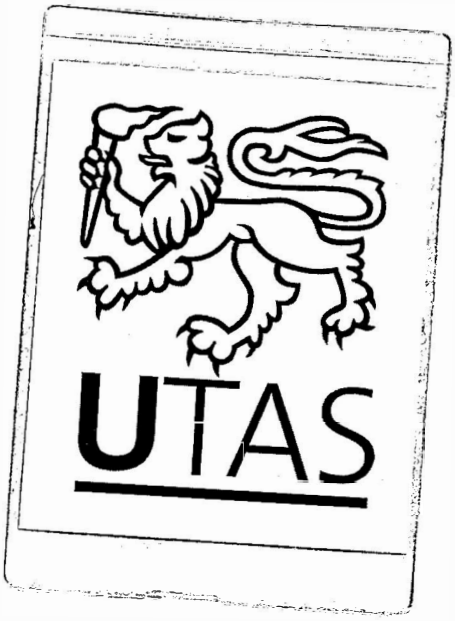
Triticum aestivum chlorotic spot virus (TACSV)

Vigna sinensis mosaic virus (VSMV)

Zea mays virus (ZMV)

5.4.3 Affinities with other groups

Plant rhabdoviruses have many similarities to vertebrate-infecting rhabdoviruses. Further study may reveal the affinity between subgroup A and members of the vertebrate-infecting *Vesiculovirus* genus may be closer than that between nucleorhabdovirus, which may have its closest affinities with members of the vertebrate-infecting *Lyssavirus* genus. However, the recent sequencing of OFV, showing OFV has a bipartite genome indicates that OFV should not be a member of the plant rhabdovirus group (Gibbs, *per. comm.*).



6. Introduction

An orchid species was first shown to contain bacilliform virus particles in 1969, described from thin sections of *Cymbidium* leaves displaying necrotic flecks in Japan (Doi *et al*, 1969). Doi *et al.* (1969) observed bacilliform virus particles measuring approximately 32 nm x 120 nm in the nuclei and cytoplasm of infected cells. The virus was designated orchid fleck virus (OFV) (Doi *et al.*, 1969). Subsequently Chang *et al.* (1973) detected bacilliform particles in several orchid genera. Chang *et al.* (1973) succeeded in the mechanical transmission and partial purification of OFV and concluded it was the same virus as described previously in Japan.

However, in 1971, Petzold (1971), and Düvel and Peters (1971) both reported bacilliform virus-like particles from *Dendrobium* sp. in Germany. Both reports were based on observations of thin sections, describing the close relationship of the intranuclear particles with the inside of the nuclear membrane and the close connection of the cytoplasmatic particles with the cytoplasmic reticulum. The intranuclear virus-like particles were unenveloped. Some enveloped particles were described by Düvel and Peters (1971) in the cytoplasm in double membrane bounded vesicles. In both reports distinctive arrangements of spokewheel inclusions were found enclosed by double membranes. Petzold (1971) tentatively named the virus Dendrobium virus, without comparing it to the virus reported earlier in Japan. Similar virus-like particles, some enveloped, with associated cytological features were found in a *Phalaenopsis lueddemanniana* from Denmark by Lesemann and Begtrup (1971). Also only detected in thin sections, the virus was tentatively named Phalaenopsis virus.

The first report of bacilliform virus-like particles detected in sap from a diseased orchid was by Begtrup (1972), from a *Dendrobium* species. Begtrup (1972) observed that only a small proportion of the particles were truly bacilliform, and the majority were bullet and rod shaped. All were unenveloped and measured 110-120 nm long and 50 nm wide. Begtrup (1972) reported the particles were only visible in crude leaf extract negatively stained with 1.2% ammonium molybdate, pH 6.8. Lesemann and Doraiswamy (1975) reported similar findings, detecting bullet-shaped particles in leaf tissue crushed in a

solution of 2% ammonium molybdate. Bullet-shaped particles were seldom observed in preparations stained with 2% phosphotungstate or uranyl acetate. However, all three stains allowed easy detection of *Cymbidium* mosaic virus and *Odontoglossum* ringspot virus.

Kitajima *et al.* (1974) completed a survey of a large collection of orchid species showing conspicuous ringspot symptoms and reported rod-like particles either in the nucleus or in the cytoplasm of infected cells. The particles were 40 nm wide and varied in length from 50-200 nm, in thin sections. Although describing them as rod-like, Kitajima *et al.* (1974) considered the particles to be similar in morphology and in their relationship to cell components to those observed in ringspot of *Phalaenopsis* and *Dendrobium* orchids in Europe (Lesemann and Begtrup 1971; Petzold 1971). Kitajima *et al.* (1974) also likened the rod-like particles observed in orchids with those associated with citrus leprosis and coffee ringspot disease.

Kitajima et al. (1974) suggested the rodlike particles observed in orchids had a viral nature due to their constant association with particular symptoms and their resemblance in morphology and intracellular localisation with rhabdoviruses. Although resembling the internal component of rhabdovirus particles, the rod-like particles appeared rarely surrounded by cytomembranes and were considered as incomplete or defective rhabdovirus particles (Kitajima *et al.* 1974). Begtrup (1972), Düvel and Peters (1971) and Lesemann and Begtrup (1971) concluded the uncoated particles seen in thin sections represented inner components of viruses related morphologically to bacilliform viruses. However, the missing coat on the particles and the unusual spokewheel-inclusions separated the viruses in *Dendrobium* and in *Phalaenopsis* from other members of the group of bacilliform viruses (Begtrup 1972; Düvel and Peters 1971).

The first report of a bacilliform virus occurring in orchids in the United States was by Ali *et al.* (1974). This was also the first report of a bacilliform virus causing floral abnormalities in orchids. The white streak symptoms described appeared distinct from other virus induced floral symptoms in orchids. Ali *et al.* (1974) reported a rhabdovirus

as well as cymbidium mosaic virus infecting the orchid, the combination of infection may have produced the colour break symptoms as neither virus alone is known to cause such symptoms. Ali *et al.* (1974) named the virus Dendrobium rhabdovirus, based on the typical bullet-shaped particle structure, together with the virus envelope and the inner component of the particles. The particle differed significantly in length and width from the bacilliform particles described in orchids by Begtrup (1972), Lesemann and Begtrup (1971) and Petzold (1971). However, it was nearly identical in size to the virus described by Düvel and Peters (1971) but showed major differences in the structural conformation of the virus particles in the infected cells. Both the particles described by Ali *et al.* (1974) and Düvel and Peters (1971) were enveloped. Lawson and Ali (1975) reported Dendrobium rhabdovirus was the first true rhabdovirus occurring in orchids. In contrast to other bacilliform viruses observed in orchids, the virus described by Lawson and Ali (1975) did not induce the formation of spoke-wheel configurations in the cytoplasm, a common feature associated with all other bacilliform viruses reported in orchids. The presence of spokewheels and the absence of enveloped particles in negatively stained preparations separates the bacilliform viruses in orchids from other rhabdoviruses, however, the morphological differences between Dendrobium rhabdovirus and other bacilliform viruses found in orchids may represent distinct stages of development of similar viruses. Lawson and Ali (1975) described both enveloped and non-enveloped particles in the perinuclear space, the presence of unenveloped particles also separates this virus from the rhabdovirus group.

Peters (1977) described a bacilliform virus from *Laelia purpuratum* and two *Laelio-Cattleya* orchids with dark red spots and ringspots. Long bacilliform particles were visible in ultrathin sections obtained from cells from the red spotted area only. The appearance of the particles showed similarities to rhabdoviruses and were enveloped, however, the characteristic properties of the virus separates it from other rhabdoviruses (Peters, 1977). The virus, named *Laelia* red leafspot virus (LRLSV) (Peters, 1977), showed some morphological similarities to the virus described by Lawson and Ali (1975), however virion length differed considerably between the two viruses.

Only Chang *et al.* (1973) succeeded in mechanically transmitting OFV, none of the other bacilliform viruses described from orchids have been sap transmitted. Further work must be done to determine if the viruses described are distinct from each other. The conflicting reports in the literature (Table 1) of the size and shape of bacilliform virions found in orchids suggests there may be more than one such virus infecting orchids. Based on their ultrastructure and their association with cell membranes, two groups may be distinguished. The first group include small bacilliform particles, approximately 100-120nm x 30nm in size. All findings have revealed that the virions are associated with the formation of "spokewheels" (Duvel and Peters, 1971; Lesemann and Begtrup, 1971; Petzold, 1971; Begtrup, 1972; Kitajima *et al.*, 1974). The other group includes bacilliform particles which are larger, 230-320nm x 80-85nm, do not form spokewheels and could possibly be true members of the rhabdovirus group.

Coffee ringspot virus and citrus leprosis virus have been compared to OFV because they are similar in particle shape and size and the particles are observed close to the inner nuclear membrane, arranged in aggregates and form "spokewheel" inclusions in the cytoplasm (Colariccio *et al.*, 1995, Lovisolo *et al.*, 1996, Chagas, 1980, Kitajima *et al.*, 1974, Kitajima *et al.*, 1972). OFV is transmitted by the mite *Brevipalpus californicus* (Maeda *et al.* 1998). Mite vectors in the genus *Brevipalpus* have also been demonstrated for coffee ringspot virus, citrus leprosis virus, ligustrum ringspot virus and green spot on passion fruit (Colariccio *et al.*, 1995, Rossetti *et al.*, 1998), suggesting the viruses may be related.

Table 9 Reported size and shape of bacilliform particles detected in orchids.

Author	**Particle shape**	**Size of particles (length x width nm)**
Lesemann & Begtrup, 1971	bacilliform bullet-shaped	110 x 30 130 or 255 x 55
Petzold, 1971	bacilliform	100 x 30
Begtrup, 1972	bacilliform bullet-shaped rod-shaped	110-120 x 50 some 100nm and 90nm
Kitajima *et al.*, 1974	rod-like	50 - 200 x 40
Lawson & Ali, 1975	bacilliform bulletshaped	320 or 180 x 85
Lesemann & Doraiswamy, 1975	bullet-shaped seldom bacilliform & cylindrical	105 x 47 100-120 x 76
Chang *et al.*, 1976	bacilliform mostly bullet- shaped	150 x 40 100-140 x 32-35 (thin sections)
Peters, 1977	bacilliform	190-230 x 80
Kondo *et al.*, 1995	bullet-shaped bacilliform	120-150 x 40

Table 10 Descriptions of symptoms induced by bacilliform particles in orchids.

Author	Orchid genera	Symptoms
Begtrup, 1972	*Dendrobium inoglossum*	coalescent yellow, chlorotic areas big dark/black ringshaped spots
Petzold, 1971	*Dendrobium* hybrid	diffuse yellow flecks, in centre brown-black necroses
Lesemann & Begtrup 1971	*Phalaenopsis lueddemanniana*	chlorotic flecks, dark necroses (mixed infection)
Lesemann & Doraiswamy, 1975	*Phalaenopsis* species *Dendrobium* species *Miltonia spectabilis* *Odontoglossum species* *Oncidium flexuosum* *Paphiopedilum* species *Stanhopea oculata* *Vanda* species	chlorotic and or necrotic leaf lesions shaped as rounded flecks or ringspots
Chang *et al.* 1976	*Angulorea* *Cymbidium* *Dendrobium* *Odontoglossum* *Oncidium* *Pescatorea*	systemic leaf chlorotic or necrotic flecks
Kitajima *et al.* 1974	*Miltonia* species *Oncidium* species *Brassica* species *Trigonidium acuminatum* *Bifrenaria harrisoniae* *Dendrobium thrysiflorum* *Phalaenopsis* hybrid *Aspasia lunata* *Hormidium fragrans*	ringspot disease
Peters, 1977	*Laelia purpuratum* *Laelio-Cattleya*	dark red spots ringspots sunken areas without colour change
Ali *et al.*, 1974	*Dendrobium phalaenopsis*	floral colour break chlorotic flecks diffuse chlorotic patches (mixed infection)
Kondo *et al.*, 1995	*Cymbidium* species	chlorotic flecks necrotic ringspots

The descriptions of symptoms induced in orchids by bacilliform viruses are varied (Table 2). These symptoms have no diagnostic value, but are distinct to the symptoms of stripes or necrotic line patterns more typical of orchids infected with *Cymbidium* mosaic virus and *Odontoglossum* ringspot virus. The large list of varied orchid genera and species (Table 2) in which bacilliform particles have been detected illustrates the bacilliform viruses have a large host range. The occurrence of bacilliform particles infecting orchids in Germany, Denmark, Japan, Korea, the United States, Brazil and Australia suggests bacilliform viruses infecting orchids are widespread in areas of the world where orchids are cultivated. The bacilliform virus particles found infecting orchids in Brazil and Europe have not been compared to those found in Japan because of insufficient information. Hence, OFV has many possible synonyms: *Dendrobium* leaf streak virus, *Dendrobium* virus, laelia red leafspot, short orchid rhabdovirus, orchid rhabdovirus, *Phalaenopsis* chlorotic spot virus, *Phalaenopsis* hybrid virus, and *Phalaenopsis* virus (Lesemann, 1986). Although the relationship of these bacilliform viruses to OFV has not been shown.

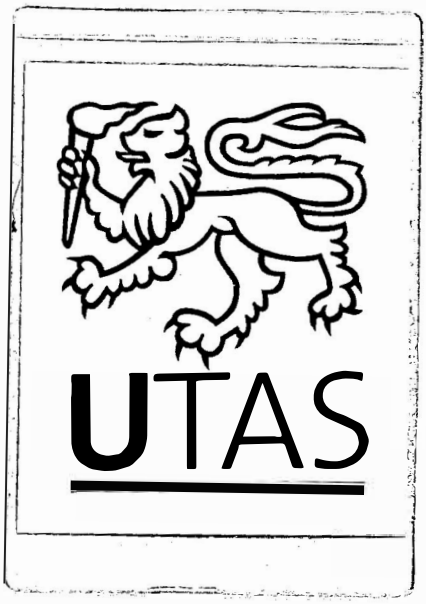
The genome structure of a Japanese isolate of OFV has been determined (Kondo *et al.*, 1998). The genome was single-stranded RNA, but unlike other rhabdoviruses, was bipartite with each component approximately 6kb in length and had a polyadenylate 3' terminus (Kondo *et al.*, 1998). The 3' and 5' terminal sequences of the two RNAs are conserved and complementary. RNA1 has 6413 nucleotides and includes five open reading frames. RNA2 has 6001 nucleotides and includes a single long open reading frame which encodes a 212 kDa protein (Kondo *et al.*, 1998). OFV is efficiently transmitted by the mite *Brevipalpus californicus* (Maeda *et al.*, 1998).

Rhabdoviruses are extremely labile and their concentration in infected plants is usually lower than that of many other plant viruses (Jackson *et al.*, 1987). Therefore, the most important factor limiting studies of plant rhabdoviruses is the difficulty of devising reproducible purification protocols suitable for recovery of adequate amounts of virus of sufficient purity for biochemical analysis (Jackson *et al.*, 1987). Diagnosis of virus

diseases are commonly conducted by enzyme-linked immunosorbent assay (ELISA) but this method is not available for use with OFV because an antisera has not been produced. Current laboratory tests used for diagnosis of OFV involve the examination of leaf sap by electron microscopy, however this is time consuming and unreliable because OFV is usually present in very low concentration. Recent studies have demonstrated the usefulness of the polymerase chain reaction (PCR) as a powerful diagnostic tool, which has the added advantage of allowing the subsequent study of genetic variation between isolates (Thomson *et al.*, 1995).

6.1 Project Objectives:

- To develop a nucleic acid based diagnostic system for OFV to provide rapid, accurate and cost effective tests which integrate with those currently available for other orchid viral pathogens commonly found and is compatible with moderate to high volume throughput of test samples.
- To evaluate the sensitivity, specificity and robustness of any such tests developed.



7. Materials and Methods

7.1.1 Virus isolates

Forty-five isolates of small bacilliform viruses were sourced from orchids of various genera, showing symptoms typical of virus infection, from private collections and nurseries in different regions in Australia, South Africa, Brazil, Germany and Hawaii (Table 11). Twenty-six Australian, and one South African isolate were provided by TASAG ELISA and Pathogen Testing Service. One isolate came from an Australian native orchid collected in the wild by Mr. Don Gowanlock. Three Brazilian isolates were obtained from Dr. Elliot W. Kitajima. Four isolates from Germany and a rhabdovirus isolate infecting an orchid from Hawaii were obtained from Dr. Dietrich-E. Lesemann. Leaf samples were either fresh, air dried, dried over calcium chloride or frozen at -80°C on receipt.

A common violet (*Viola* sp.) showing areas of leaf chlorosis and containing OFV-like particles, observed by electron microscopy, was supplied by Mr. Don Gowanlock (Table 12). An isolate of coffee ringspot virus (CoRSV) was obtained from Mr. Cesar M. Chagas in Brazil (Table 12). Another isolate of CoRSV, an isolate of citrus leprosis virus and schefflera, hibiscus, ivy and ligustrum leaves showing ringspot symptoms were obtained from Dr. Elliot W. Kitajima, also in Brazil (Table 12).

7.2 Isolation & Characterisation of Virions & Viral Nucleic Acids

7.2.1 Electron microscopy

Electron microscopy was used for partial characterisation of the bacilliform OFV virion structures. Expressed leaf sap mounted directly onto Parlodion and carbon coated, copper/rhodium 400 mesh square grids were negatively stained with ammonium molybdate, pH 5.2 and examined with a transmission electron microscope (Phillips EM 201). Morphological characterisation of isolates was made based on particle dimensions.

Table 11 List of OFV isolates compiled, including sample number, location of collection and orchid genera in which OFV was detected.

Sample no.	Country (State)	Genera
003	Hillman, Western Australia	*Dendrobium* sp.
005	Brazil	*Miltonia mourelliana*
007	Brazil	*Miltonia rignelli x mourelliana*
009	Brazil	*Hormidium calamarium*
011	Bairnsdale, Victoria	*Cymbidium* sp.
013	Australian Capital Territory	*Cattleya sp.*
017	Kings Langley, New South Wales	*Dendrobium* sp.
019	Cromer, New South Wales	*Cymbidium* sp.
023	Germany	*Wilsonara* hybrid
024 *	Gladesville, New South Wales	*Cymbidium* hybrid
025	Hornsby, New South Wales	*Dendrobium* sp.
027	Blacktown, New South Wales	*Cymbidium* sp.
029	Belmont, New South Wales	*Dendrobium* sp.
031	Sydney, New South Wales	*Bulbophyllym elbertii*
033	Sydney, New South Wales	*Dendrobium hybrid*
037	Lenah Valley, Tasmania	*Oncidium* sp.
039	Howrah, Tasmania	*Oncidium* sp.
041	Australian Capital Territory	*Cymbidium hybrid*
043	Smithfield, New South Wales	*Cymbidium* sp.
045	Germany	*Cymbidium* sp.
047	Germany	unknown species
052	Germany	unknown species
053	Durban, South Africa	*Cymbidium* sp.
056	Brisbane, Queensland	*Dendrobium kingianum* (Australian native)

057***	St. Agnes, South Australia	*Cymbidium* sp.
058***	North Richmond, New South Wales	*Cattleya* sp.
059*	Blacktown, New South Wales	*Cymbidium* hybrid
060*	Belmont, New South Wales	*Dendrobium* hybrid
061*	Kings Langley, New South Wales	*Cymbidium* sp.
062*	Hornsby, New South Wales	*Cymbidium* sp.
063*	Bellingen, New South Wales	*Dendrobium* hybrid
064*	Gracemere, Queensland	*Cymbidium* sp.
077**	Hawaii	*Epidendrum* sp.
237	Brisbane, Queensland	*Cymbidium* sp.
494	New Town, Tasmania	*Cymbidium hybrid*
590	Burra Creek, New South Wales	*Baptistonia echinata*
591	Burra Creek, New South Wales	*Angracum sesquipedale*
593	Burra Creek, New South Wales	*Stanhopea embreii*
770***	Sydney, New South Wales	*Dockrillia* hybrid
773	Sydney, New South Wales	*Cattleya aclandii*
775	Sydney, New South Wales	*Dendrobium fimbriatum*
776	Sydney, New South Wales	*Liparis plantaginea*
777	Sydney, New South Wales	*Maxillaria striata*
778	Sydney, New South Wales	*Odontoglossum* hybrid
780	Australian Capital Territory	*Hamelwellsara* hybrid

*air dried sample shown to be positive for OFV by electron microscopy but failed to yield a PCR product using mN2 and polydT/SP6 primers.

**sample is infected by an enveloped bacilliform particle distinctly larger than OFV and failed to yield a PCR product using mN2 and polydT/SP6 primers.

***sample detected by RT-PCR however was not sequenced.

Table 12 List of small bacilliform isolates from other plants, including sample number and location of collection.

Sample no.	Plant type	Location
090	*Viola* sp.	Qld, Australia, D. Gowanlock
091	Coffee	Brazil, C. M. Chagas
092	Coffee	Brazil, E. W. Kitajima
093	Ligustrum	Brazil, E. W. Kitajima
094	Ivy	Brazil, E. W. Kitajima
095	Hibiscus	Brazil, E. W. Kitajima
096	Schefflera	Brazil, E. W. Kitajima
097	Citrus	Brazil, E. W. Kitajima

7.2.2 Mechanical Inoculation

Mechanical transmission of OFV was attempted in order to introduce OFV to a herbaceous host to aid virion purification, and to test experimental range. OFV isolate 494 was maintained in *Cymbidium* sp. grown under glasshouse conditions, with an average temperature range of 20-25°C. The virus was inoculated to uninfected seedlings, grown under glasshouse conditions, with an average temperature range of 20-25°C, with 4 to 6 expanded leaves. Nine herbaceous species were challenged belonging to the *Chenopodiaceae, Solanaceae* and *Aizoaceae* families. Sixty seedlings of each *Chenopodium quinoa, C. murale, C. amaranticolor, C. foliosum* and *C. alba* were challenged four times. Twenty seedlings of *each Nicotiana glutinosa, N. Tabacum* 'White Burley', *Tetragonia expansa,* and *Petunia hybrida* were challenged four times. Challenges were also made to six *Cymbidium* back bulbs, eight times. Inoculum was prepared by grinding 1g of symptomatic leaves using a mortar and pestle, in 20 ml of cold buffer. Either 0.01M phosphate buffer, pH 7.2 (Appendix 1) or 0.02M HEPES buffer, pH 7.4 (Appendix 1) was used. 1% celite was added and the homogenate was rubbed onto the leaves of host plants using sterilised cotton buds. Leaves were rinsed after five minutes. The freshly inoculated host plants were kept in the dark for 2 hours before being transferred to the glasshouse. Symptoms were recorded 10-14 days after inoculation and the source plants and inoculated host plants were examined with electron microscopy to determine infection with OFV.

7.2.3 Virion purification

Primarily the method described by Chang *et al.* (1976) for the partial purification of OFV was used. Approximately 35g of symptomatic *Cymbidium* leaves or *Chenopodium quinoa* 15-23 days post-inoculation, were freshly harvested and homogenised in 3, 4 or 5 volumes of extraction buffer (Appendix 1) using a Waring commercial blender (Waring products division, New Hartford, USA) at 4°C. The homogenate was then strained through two layers of muslin cloth and the filtrate was clarified by centrifugation at 5,000 rpm for 15 minutes at 4°C in a Sorvall RC5C, SS-34 rotor (Dupont Instruments, Northside, NSW). The supernatant was collected and centrifuged at 30,000 rpm in a Beckman L8-M ultracentrifuge for 2 hours, at 4°C. The pellet was resuspended in 0.1M phosphate buffer, pH 7.0, using in a glass tissue homogenizer, and centrifuged at 5,000 rpm for 15 minutes at 4°C. The supernatant was centrifuged at 30,000 rpm for 2 hours at 4°C. The pellet was resuspended in 0.1M phosophate buffer, pH 7.0. Following each step of differential centrifugation the purified preparations were examined for the presence of virus particles and the amount of contaminating host material present was assessed by electron microscopy. Virion preparations were placed on carbon-stabilized copper grids, negatively stained with ammonium molybdate, pH 5.2 and viewed in a transmission electron microscope. Subsequently, the virion preparation was run through another cycle of differential centrifugation if the virion preparation was deemed viable through assessment with electron microscopy.

Additional modifications incorporating a number of rhabdovirus purification methods (Hunter *et al.* 1990, Jackson and Christie,1977, Creamer, 1992) were attempted twice in order to improve the success of purifications. The extraction buffer described by Hunter *et al.* (1990) (Appendix 1) was used, the homogenate was squeezed through four layers of muslin cloth and centrifuged at 5,000 rpm for 15 minutes at 4°C. The supernatant was layered on a step gradient formed from 8ml of 300mg/ml of sucrose and 5ml of 600mg/ml of sucrose in extraction buffer at pH 7.4 (Hunter *et al.*, 1990). The extraction

buffer of Jackson & Christie (1977) (Appendix 1) was used and the supernatant collected after centrifugation at 5,000 rpm for 10 minutes at 4°C was layered over a discontinuous sucrose gradient of 300 and 600 mg/ml in extraction buffer adjusted to pH 7.5 (Jackson & Christie, 1977). One gram of Celite was added to the virus preparation after differential centrifugation according to Cremer (1992).

Samples of purified virus particles were stored at 4°C in 0.1M phosphate buffer, pH 6.8 with 1% glutaraldehyde.

7.2.4 Extraction of Viral RNA

Following successful partial purification of OFV the viral RNA was extracted from three different suspensions using an RNeasy Plant Mini kit (QIAGEN) according to the manufacturers instructions. RNA was also extracted from partially purified suspensions of OFV using the method of Thomson *et al.* (1995). Three different samples were combined and incubated for 30 minutes with 0.1% sodium dodecyl sulphate and 50 µg/ml proteinase K. The pooled sample was then emulsified with an equal volume of phenol saturated with 0.1M Tris-HCl, pH 8.0, the suspension was centrifuged and the supernatant was mixed with an equal volume of phenol:chloroform (1:1). Following centrifugation the supernatant was mixed with an equal volume of chloroform:n-amyl alcohol (24:1). RNA was precipitated from the aqueous phase by the addition of 0.1 volume of 3M sodium acetate, pH 5.2 and 2.5 volumes of cold ethanol and incubation at -20°C for 3 hours. The sample was centrifuged at 10,000g for 30 minutes at 4°C, pelleted nucleic acid was washed with 70% ethanol and dried before being resuspended in 30 µl sterile water. This method was repeated on two other occasions.

Samples of nucleic acids resulting from the RNA extracts were electrophoresed on 1% agarose gels, both following digestion with RNase A (Amersham Pharmacia Biotech) and untreated.

7.3 Cloning of Viral Nucleic Acids

7.3.1 cDNA synthesis

10µl of viral RNA extracted from purified OFV particles using the method of Thomson *et al.* (1995), was diluted with 10µl sterile, DEPC treated water, heated at 65°C for 10 minutes, then chilled on ice. The RNA solution was added to the first strand reaction mix of a TimeSaver cDNA synthesis kit (Pharmacia Biotech) with the random hexamer primer diluted at 1/200. The complete protocol was followed according to the manufacturers instructions, including the addition of EcoR1/Not1 adapters. The cDNA was then ligated directly into a pUC 18 vector, using a Ready to Go pUC 18 EcoR1/BAP + Ligase kit (Pharmacia Biotech). 20µl of cDNA was added and the protocol was followed according to the manufacturers instructions. The ligation reaction was performed with both heat inactivation by heating at 70°C for 10 minutes and without heat inactivation of the ligation reaction.

7.3.2 Transformation of chemically competent cells.

100µl of *Epicurian coli* Sure 2 supercompetent cells (Stratagene) were thawed on ice. 1 µl of the ligation mix was added and incubated on ice for 10 minutes. The cell suspension was heat-shocked at 42°C for 30 seconds, then chilled on ice for 2 minutes. 900µl of 42°C preheated Loria Broth (LB) liquid (Appendix 1) was added and incubated at 37°C with shaking for 1 hour. Dilutions of the cell suspension were plated onto selection LB containing ampicillin (50µg/ml) and 40µl of each 20 mg/ml stock solution of IPTG (β-D-thiogalactopyranoside) and XGal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) per plate.

1µl of the ligation mix was also added to 100µl DH5α *E. coli* thawed on ice, and incubated for 10 minutes on ice. The cell suspension was heat-shocked at 42°C for 90 seconds and then chilled on ice for 2 minutes. 450µl LB liquid, preheated to 37°C was

added and the cell suspension incubated at 37°C with shaking for 1 hour. 0.8mg XGal and IPTG was spread onto the surface of each selection LB plate containing ampicillin (50µg/ml) before dilutions of the cell suspension were plated out.
Individual, white colonies growing on the LB + ampicillin plates containing IPTG and XGal were placed into 2 ml of liquid LB and incubated overnight with shaking at 37°C. Individual colonies were also placed onto a master plate and numbered.

7.4 Sequence Analysis of Cloned Nucleic Acids

7.4.1 Miniprep of plasmid DNA

Minipreparations of plasmid DNA were obtained by the alkaline lysis method described by Sambrook *et al.* (1989). 1.5ml of a single colony culture was centrifuged at 13,000rpm for 30 seconds at 4°C in a microfuge. The bacterial pellet was resuspended in 100µl of ice-cold Solution I (Appendix 1) with vortexing. 200µl of Solution II (Appendix 1) was added, mixed by inverting the tube rapidly, then stored on ice. 150µl of Solution III (Appendix 1) was added, and vortexed in an inverted position. The solution was stored on ice for 3-5 minutes, then centrifuged at 13,000rpm for 5 minutes at 4°C. The supernatant was mixed with an equal volume of phenol:chloroform (1:1). After centrifugation at 13,000rpm for 2 minutes at 4°C the supernatant was mixed with 2 volumes of ethanol at room temperature. The DNA was precipitated for 2 minutes at room temperature before centrifuging at 13,000rpm for 5 minutes at 4°C. The pellet was washed with 1ml of ice cold 70% ethanol and air dried. The pellet was redissolved in 50µl of TE, pH 8.0 containing 20µg/ml RNase A. Minipreparations of plasmid DNA were also obtained using the QIAprep Spin plasmid kit (QIAGEN) following the manufacturers instructions.

Plasmid DNA was screened using restriction enzyme digests. 3µl of plasmid DNA was cut with 1µl Not 1 restriction enzyme in 1µl Buffer H (Boehringer Mannheim,

Biochemica) and 5µl water, the solution was incubated at 37°C for 1 hour before being visualised on a 1% agarose gel.

7.4.2 Sequencing Plasmid inserts

Plasmid DNA containing an insert was sequenced using 8µl BigDye Terminator Ready Reaction Mix (PE Applied Biosystems), 0.32µl of 10µM universal forward primer (Clontech laboratories Inc.), 4µl of plasmid DNA template and 7.68µl water to make a total volume of 20µl each reaction. The following thermocycle regime was used, repeated for 25 cycles: 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The extension products were purified by pipeting each reaction mixture into a solution of 2µl of 3M sodium acetate, pH 5.0 and 50µl of 100% ethanol, stored on ice for 10 minutes before centrifugation at 13,000rpm for 30 minutes at 4°C. The pellet was rinsed with 250µl of 70% ethanol and dried in a vacuum centrifuge.

7.5 Development of RT-PCR Detection System

7.5.1 Nucleic acid extraction

Four methods were used for the extraction of viral RNA from healthy and viral infected orchid leaf tissue for use in a reverse transcription - polymerase chain reaction (RT-PCR) system. (1) RNA was extracted from leaf tissue using an RNeasy Plant Mini kit (QIAGEN) according to the manufacturers instructions. (2) Total plant RNA was extracted by grinding 2-3g of leaf material in liquid nitrogen and adding the ground material to 15ml of CTAB buffer (Appendix 1) warmed to 65°C and incubated for 10 minutes. 2 volumes of chloroform:isoamyl alcohol (24:1) was added and the solution centrifuged at 10,000 rpm for 5 minutes. The supernatant was removed and ¼ volume of 10M LiCl and 2 volumes of ethanol were added. RNA was precipitated overnight at 4°C.

The solution was centrifuged for 20 minutes at 4°C. The pellet was washed in 70% ethanol and air dried then resuspended in 30-50µl sterile, DEPC treated water.

Total nucleic acids were extracted from leaf tissue as described by (3) Mackenzie *et al.* (1998). Approximately 100mg of fresh or dried leaf tissue was frozen in liquid nitrogen and ground to a fine powder. 500µl wash buffer (Appendix 1) was added and mixed by vigorous vortexing. Samples were centrifuged at 10,000g for 5 minutes and nucleic acids were extracted from the resultant pellet by adding 600µl CTAB buffer (Appendix 1), the solution was mixed thoroughly and incubated at 55°C for 15-30 minutes. The mixture was shaken with 300µl chloroform:isoamyl alcohol (24:1), and centrifuged at 14,000g for 5 minutes. The aqueous phase was mixed with 0.1 volume 7.5M ammonium acetate and 1 volume isopropanol, following precipitation at -20°C for 45 minutes and centrifugation at 14,000g for 20 minutes at 4°C, the pellet was washed with 70% ethanol and dried. The total nucleic acids were resuspended in 30-50µl sterile, DEPC treated water.

Total nucleic acids were also extracted from leaf tissue as described by (4) Braithwaite *et al.* (1995). Approximately 50mg of leaf was frozen and ground in liquid nitrogen. 500µl STE buffer (Appendix 1), containing 50mg/ml polyvinylpolypyrrolidone, and 60µl of 10% SDS was added and mixed. 500ul phenol/chloroform/isoamyl alcohol (25:24:1) and 0.1 volume 3M sodium acetate (pH 5.2) was then added. The sample was mixed by inverting the tube and centrifuged at 10,000g at 4°C for 10 minutes. The aqueous phase was re-extracted with an equal volume of phenol/chloroform/isoamyl alcohol and 0.1 volume 3M sodium acetate. Highly coloured samples were extracted a third time with phenol/chloroform/isoamyl alcohol. Nucleic acids were precipitated from the final aqueous phase with 0.1 volume 3M sodium acetate and 2 volumes ethanol. After precipitation at -20°C for 1 hour the samples were centrifuged at 15,000g at 4°C. The pellet was washed with cold 70% ethanol, dried and nucleic acids were resuspended in 30-50µl sterile, DEPC treated water.

7.5.2 Reverse transcription-polymerase chain reaction and sequencing

Dr Hideki Kondo and Professor Tetsuo Tamada of Okayama University, Japan, kindly supplied PCR primer sequences derived from genes adjacent to the 3' end of both genomic sequences of the bipartite Japanese OFV isolate; one, mN2 primer (5'-TGCAGGAATATAGCCGACATGTT-3'), in the gene encoding the nucleoprotein, and the other, mP1 primer (5'- TATATTCTCATTCCAGGGA -3'), the phosphoprotein. RT-PCR was done using either a one step or two step reaction system. One step RT-PCR was done in a 25 µl reaction mix using the SuperScript One-Step RT-PCR System (Life Technologies, Gibco BRL). Empirical testing with $MgCl_2$ final concentrations of 2mM, 3mM, 4mM and 5mM determined the optimum concentration of $MgCl_2$. 0.5 µl of extracted nucleic acid was used with 15 pmol of a polydT/SP6 primer (5'-AACTGGAAGAATTCGCGGCGGCAGGAATTTTTTTTTTTTTTTTT-3'), which is complimentary to the polyadenylated region at the 3'-terminus of the OFV genome with a SP6 bacteriophage primer attached and 50ng of the mN2 primer or the mP1 primer. The following thermocycle regime was used; 45°C for 30 min, 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 56°C for 45 sec, 72°C for 1 min, finally 72°C for 10 min.

The two step reaction was done with an initial reverse transcription step in a 10µl reaction mix containing 1µl 10x Reaction buffer IV (Applied Biosystem), 1µl each of dATP, dTTP, dGTP and dCTP (10mM, Perkin Elmer), 0.5µl AMV Reverse trancriptase (Pharmacia Biotech), 0.5µl RNAguard RNase inhibitor (Porcine, Pharmacia Biotech), 1µl polydT/SP6 primer (30pmol/µl), 0.5µl sterile, DEPC treated water, 2µl 25mM $MgCl_2$ solution (Perkin Elmer) and 0.5µl of extracted nucleic acid. The solution was held at 45°C for 30 minutes, the second reaction mix containing 8µl 25mM $MgCl_2$ solution (Perkin Elmer), 4µl 10x Reaction buffer IV (Applied Biosystem), 0.25µl Taq DNA polymerase (Applied Biosystem), 1µl mN2 or mP1 (50ng) primer and 26.25µl sterile water was then added, to bring the total volume of the reaction mixture to 50µl. The following thermocycle regime was used; 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 56°C for 45 sec, 72°C for 1 min, finally 72°C for 10 min. Empirical testing

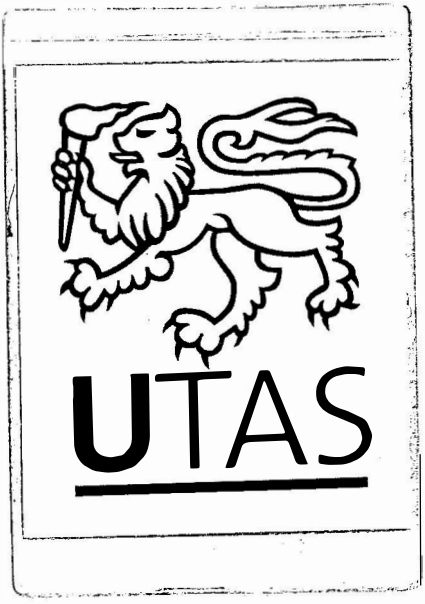
with $MgCl_2$ final concentrations of 2mM, 3mM, 4mM and 5mM determined the optimum concentration of $MgCl_2$. The cycling protocol was altered from that recommended by Dr. Hideki Kondo, which was 45°C for 30 min, 94°C for 1 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min and a final incubation of 72°C for 10 minutes.

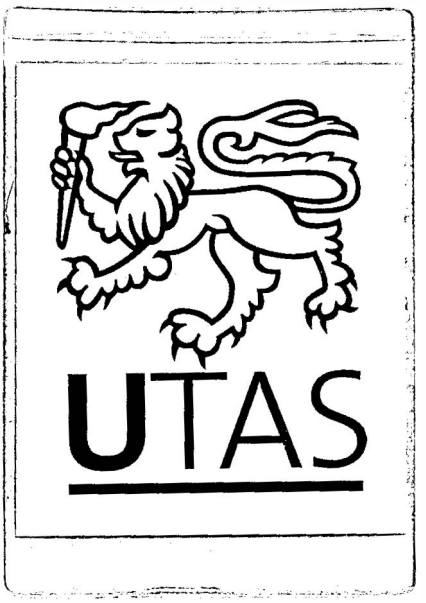
DNA fragments in a 5 µl aliquot of product mix were fractionated by electrophoresis in 1% agarose gels containing ethidium bromide. Amplified fragments of DNA (800bp) from the remaining product mix were excised from the agarose gel without exposure to UV light and purified using the QIAquick Gel Extraction Kit (QIAGEN) as recommended by the manufacturers. DNA concentration was estimated after further agarose gel electrophoresis and comparison against a DNA mass ladder (Life Technologies, Gibco BRL), and 10-40 ng of each purified PCR product was sequenced in both directions using the SP6 or mN2 primers and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Sequencing was done by the Biomolecular Resource Facility at the John Curtin School of Medical Research, Australian National University.

7.5.3 Computer analyses

All sequences were checked against the international nucleotide sequence databases using the BLAST programs supplied by the Australian National Genomic Information Service. Thirty-four OFV sequences were aligned and the longest open reading frame of each used for comparisons; sequences to the 3' side of the first stop codon were deleted from each, and the 660 nucleotide 5' ORF used in the comparisons. Aligned sequenced were used to calculate and draw a neighbor-joining tree representing their relationships. The relationships of the OFV sequences were determined from the pairwise % nucleotide differences of the aligned sequences (aligned by CLUSTAL V) using the neighbor-joining method. To assess the certainty of the calculated relationships, the same alignments were translated into amino acids and these sequences were compared by a maximum likelihood

method. This was done by quartet puzzling using the program PUZZLE version 4 (Strimmer and von Haeseler, 1996) after all sequence gaps had been excluded. Likelihoods were calculated using the HKY substitution model (Hasegawa *et al.*,1985) and a gamma distribution of rates of change for variable sites with a shape parameter estimated from the data using a neighbor-joining tree.





8. Results

8.1.1 Virus isolates

Leaves of orchids infected with a bacilliform virus showed chlorotic flecks, ringspots and/or necrotic spots (Figure 2) of varying severity. Symptoms differed between orchid genera. Plants co-infected with other viruses showed more severe symptoms (Figure 3). Floral symptoms were not observed because the majority of samples collected included only leaves.

8.1.2 Electron Microscopy

The OFV isolates examined had non-enveloped, bacilliform and bullet-shaped particles measuring between 70-120 x 30-50nm in dip preparations (Figure 1). Particles often appeared in tandem or longer formations and short, broken particles were also observed. Complete particle structure was difficult to observe because of the fragility of the particles.

Figure 1 Electron micrograph of OFV particles (1cm = 40nm) in expressed leaf sap preparation, negatively stained using ammonium molybdate, pH 5.2.

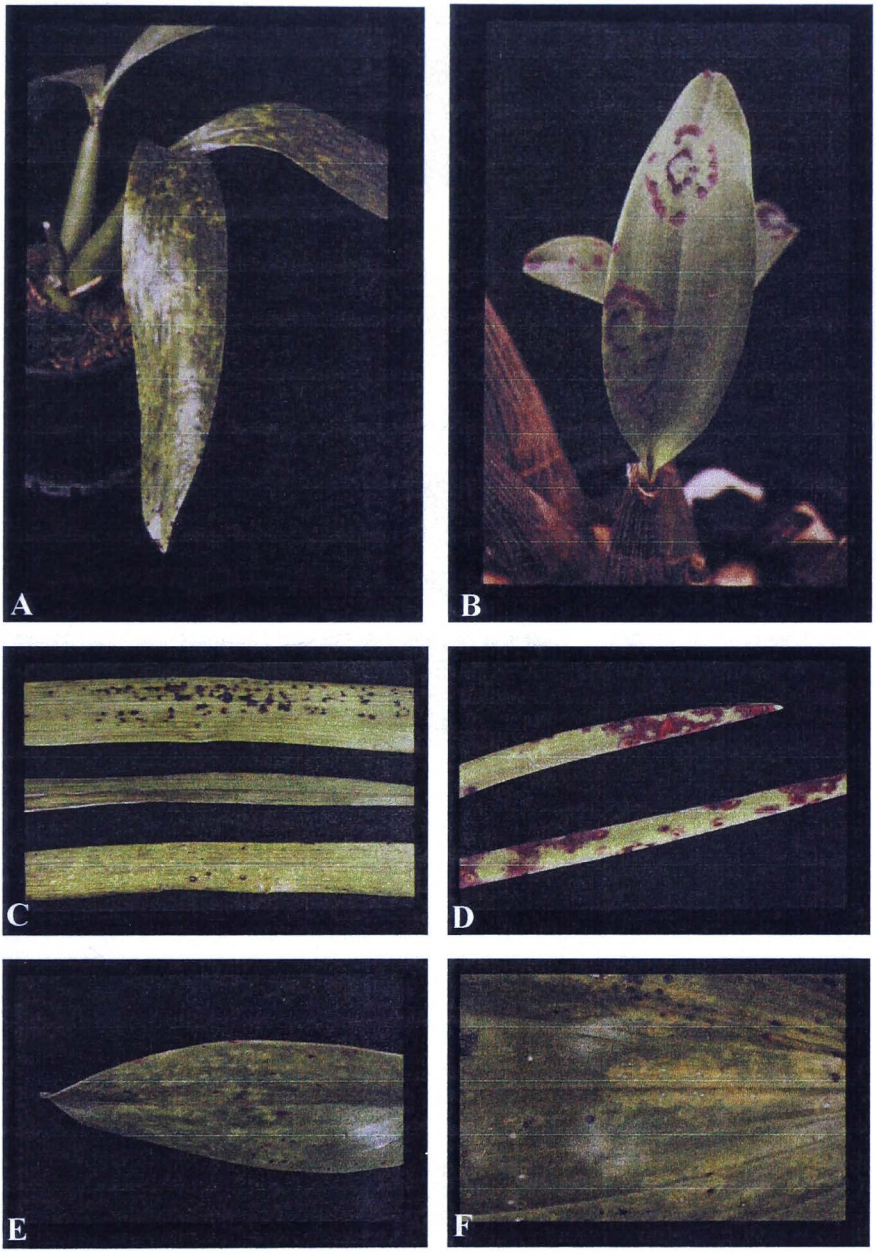


Figure 1 Different orchid genera showing a variety of symptoms induced by OFV infection. **A**. Angracum sp., **B**. Dendrobium sp., **C**. Cymbidium sp. **D**. Dockrillia hybrid, **E**. Odontoglossum hybrid, **F**. Stanhopea sp.

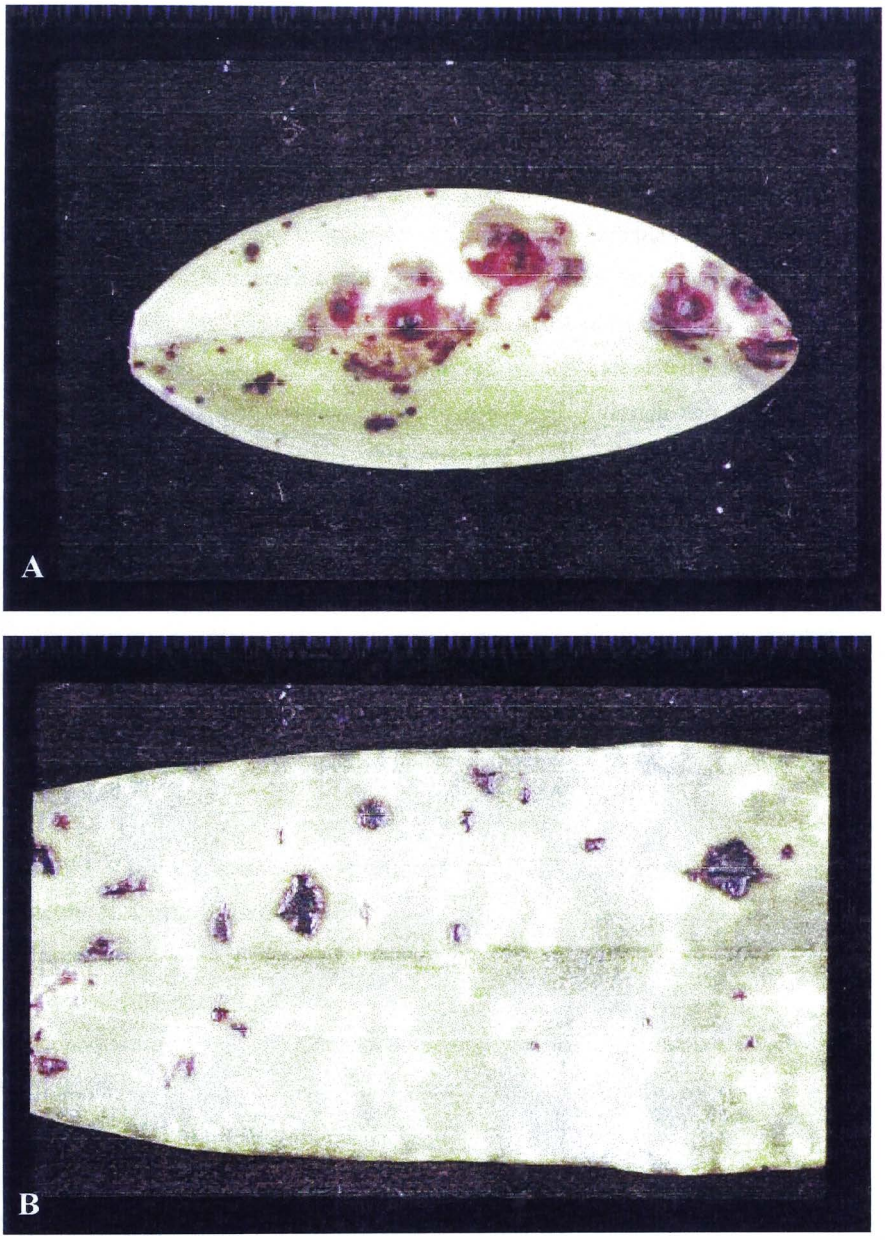


Figure 2 **A. Cattleya sp. infected with OFV and a potex virus.**
B. Dendrobium sp. infected with OFV and a potex virus.

8.1.3 Mechanical Inoculation

The two inoculation buffers used resulted in comparable infection rates. The success of mechanical inoculation varied with season. Late spring, summer and early autumn was the best time for mechanical inoculation. During summer around 80-90% of inoculations resulted in successful infections with OFV. When the temperature in the glass house was cool (<20°C) and day length relatively short, only 0-10% of inoculations were successful. Local chlorotic mosaic patterns were observed on the inoculated leaves of *Nicotiana glutinosa*, *Chenopodium amaranticolor* (Figure 4), *C. foliosum* and *C. alba* after 2-3 weeks. No systemic symptoms were observed (Table 13). Chlorotic spots were observed on the inoculated leaves of *Chenopodium quinoa* (Figure 5) and *C. murale* after 2-3 weeks. Chlorotic spots were observed on the upper leaves 1 week later, indicating a systemic infection (Table 13). Electron microscopy examination confirmed the presence of bacilliform particles in the inoculated leaves of *N. glutinosa, C. amaranticolor, C. foliosum* and *C. alba* and in the upper leaves of *C. quinoa* and *C. murale.*
Mechanical inoculation to *Tetragonia expansa, Petunia hybrida, Nicotiana tabacum* 'White Burley' (Table 13) and *Cymbidium* back bulbs was not successful.

Table 13 Results of challenge experiments with OFV to alternate hosts.

Systemic susceptible plant species	
Chenopodiaceae	*Chenopodium murale, C. quinoa*
Local susceptible plant species	
Chenopodiaceae	*Chenopodium amaranticolor, C. foliosum, C. alba*
Solanaceae	*Nicotiana glutinosa*
Non-susceptible plant species	
Aizoaceae	*Tetragonia expansa*
Solanaceae	*Petunia hybrida, Nicotiana tabacum* 'White Burley'

Figure 3 Local infection on mechanically inoculated leaf of *C. amaranticolor* by OFV.

Figure 4 Systemic infection by OFV of mechanically inoculated *Chenopodium quinoa*

8.1.4 Virus purification

The extraction method of Chang *et al.* (1976) resulted in low yields of OFV virions. Virions were not observed in all purification attempts, particles often degraded, however the initial volume of extraction buffer used did not influence this result. The amount of contaminating host material present after each differential centrifugation step varied little. Host materials could not be removed in later steps without destroying the virus particles. Therefore, virus preparations that were free of host contaminants were not obtained. Using the rhabdovirus purification methods of Hunter *et al.* (1990), Jackson & Christie (1979) or Creamer (1992) also failed to purify OFV virions and did not improve the initial method of Chang *et al.* (1976). Therefore, direct production of OFV sera was not possible through particle purification but enabled attempts at cloning viral nucleic acids. Electron microscopy of each of the fractions collected at each of the differential centrifugation steps during virus purification visualised virions that were similar in size to those observed from leaf dip preparations.

8.1.5 Extraction of viral RNA from partially purified virus preparations

No detectable RNA was retrieved from the RNeasy Plant Mini kit (QIAGEN) from three preparations of partially purified OFV. However, a 1kb band of nucleic acid was visualised on 1% agarose gels from RNA extracts from two preparations of partially purified OFV following the method of Thomson *et al.* (1995). The bands were digested by RNase A, suggesting they were RNA.

8.1.6 cDNA synthesis

Restriction enzyme digests and sequencing of the plasmid DNA showed the plasmids isolated from white transformed colonies did not contain inserts of the cDNA, but rather

contained multicopies of the plasmids annealed to each other (Figure 6), which is a common problem when cDNA template is present in low concentrations.

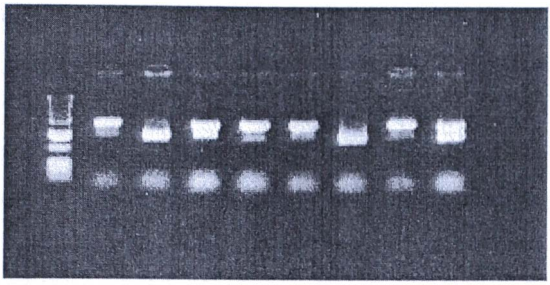


Figure 6 Plasmid DNA on 1% agarose gel, showing the plasmids did not contain inserts of the cDNA.

8.2 Development of RT-PCR Detection System

8.2.1 Nucleic Acid extraction from leaf material for RT-PCR

Extraction of RNA from leaf tissue using an RNeasy Plant Mini kit (QIAGEN) and total RNA using a CTAB buffer did not yield amplified fragments when tested using RT-PCR and known positive samples. The methods of total nucleic acid extraction by Mackenzie *et al.* (1998) and Braithwaite *et al.* (1995) were both successful, however the method of Mackenzie *et al.* (1998) yielded amplified products of greater concentration in RT-PCR tests, especially for dried samples.

8.2.2 Reverse transcription-polymerase chain reaction and sequencing

RT-PCR using primers mP1 and polydT/SP6 did not give any amplified products from any samples. RT-PCR using primers mN2 and polydT/SP6 amplified a single product of 800 bp from all OFV isolates except seven of the nineteen air dried samples, although

they were known to contain OFV virions (Figure 7). RT-PCR tests of healthy orchid material did not yield any amplified products. RT-PCR tests of the native orchid collected from the wild also produced a 800 bp amplicon confirming infection with OFV. The orchid sample from Hawaii containing rhabdovirus virions larger than those of OFV gave no DNA fragment when tested using RT-PCR and the mN2 and polyA/SP6 primers.

The optimum concentration of $MgCl_2$ was determined to be 5mM for both one and two step RT-PCR reactions. Altering the cycling conditions from those recommended by Dr. Hideki Kondo by increasing the denaturing time by 30 seconds, increasing the annealing temperature from 50°C to 56°C, decreasing the extension time by one minute and increasing the number of cycles from 30 to 35 gave optimum results for both the one and two step RT-PCR reactions. RT-PCR using a one step or two step reaction gave equivalent product qualities.

The 800 bp amplified fragments were sequenced. Multiple sequence alignment revealed that the sequences of the Australian, Brazilian and two of the German isolates of OFV were identical or very similar (Appendix 2). Within these isoates there was some indication that they may fall into two sub-groups, with only 2.5% sequence divergence (Figure 8). Each putative subgroup contained isolates collected from Australia, Brazil and Germany. The third isolate from Germany represented a second distinct strain of OFV, with 16% sequence divergence (Figure 8). The Japanese isolate is not included in this study as insufficient sequence was supplied by Dr. Hideki Kondo.

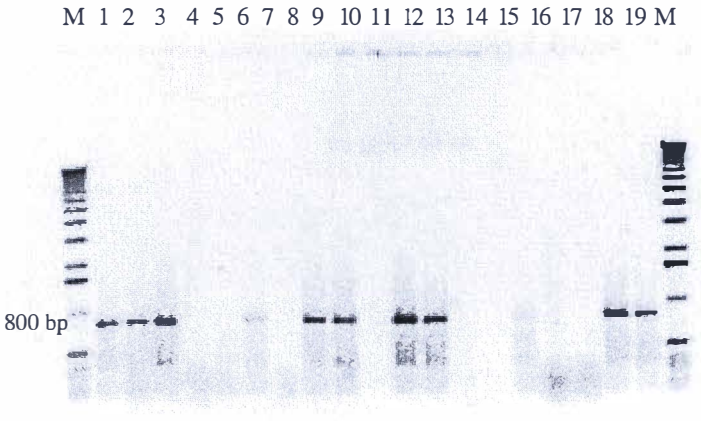


Figure 7 Positive and negative RT-PCR reactions conveyed from fresh, frozen and air dried orchid leaves shown to be infected with OFV by electron microscopy. Lanes 1,2,3, 6 were frozen samples. Lanes 8,9,11,12,15,18,19 were fresh samples. Lanes 4,5,7,10,13,14,16 were air dried samples. Lane 17 was a known healthy sample.

Neighbor-joining trees and maximum likelihood trees of amino acid sequences were compared visually and found to be similar in their major topological features - the nodes, that were identical in the OTUs they linked, are circled (Figure 8).

A search of the international nucleotide database with the OFV sequence showed it to be distantly related to those of three cytorhabdovirus sequences (Figure 9), notably lettuce necrotic yellows cytorhabdovirus (Appendix 2).

A single product of 800bp was amplified from one isolate of coffee ringspot virus using the mN2 and polydT/SP6 primers. The product was shown to be identical to OFV when sequenced. RT-PCR tests and sequencing showed the common violet also contained an isolate of the most commonly found OFV strain. The other CoRSV isolate, citrus leprosis virus isolate, schefflera, hibiscus, ivy and ligustrum leaves did not produce amplified products when tested using RT-PCR with the OFV primers.

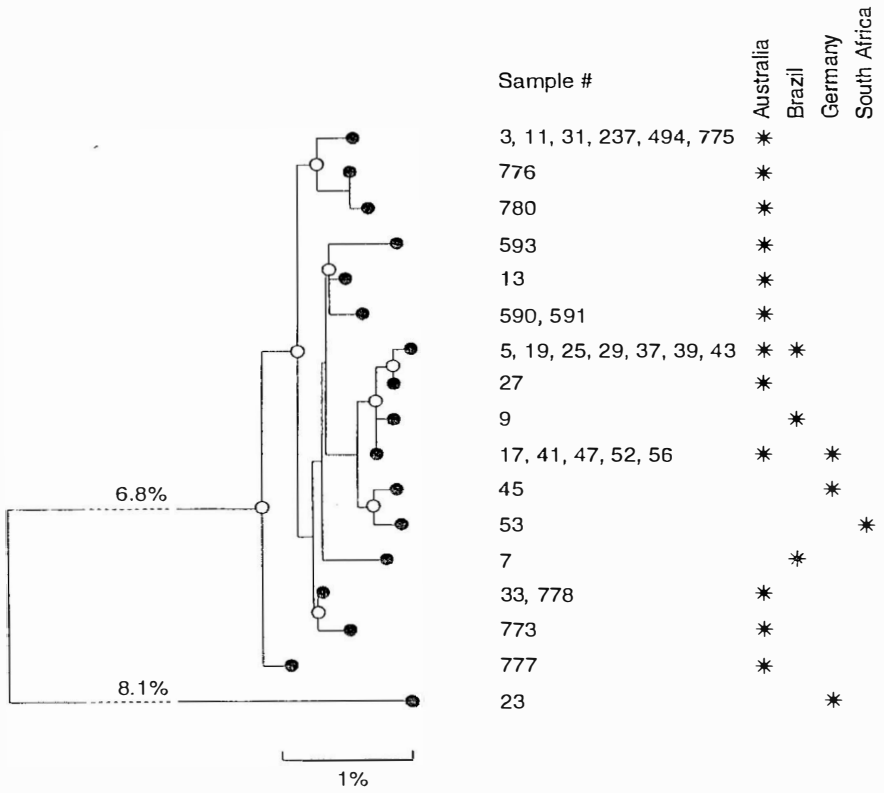


Figure 8 A neighbor-joining tree calculated from the % differences between the aligned nucleotide sequences encoding the nucleoprotein of 34 OFV isolates. Nodes representing clusters found to be the same in the neighbor-joining and maximum likelihood analyses are circled.

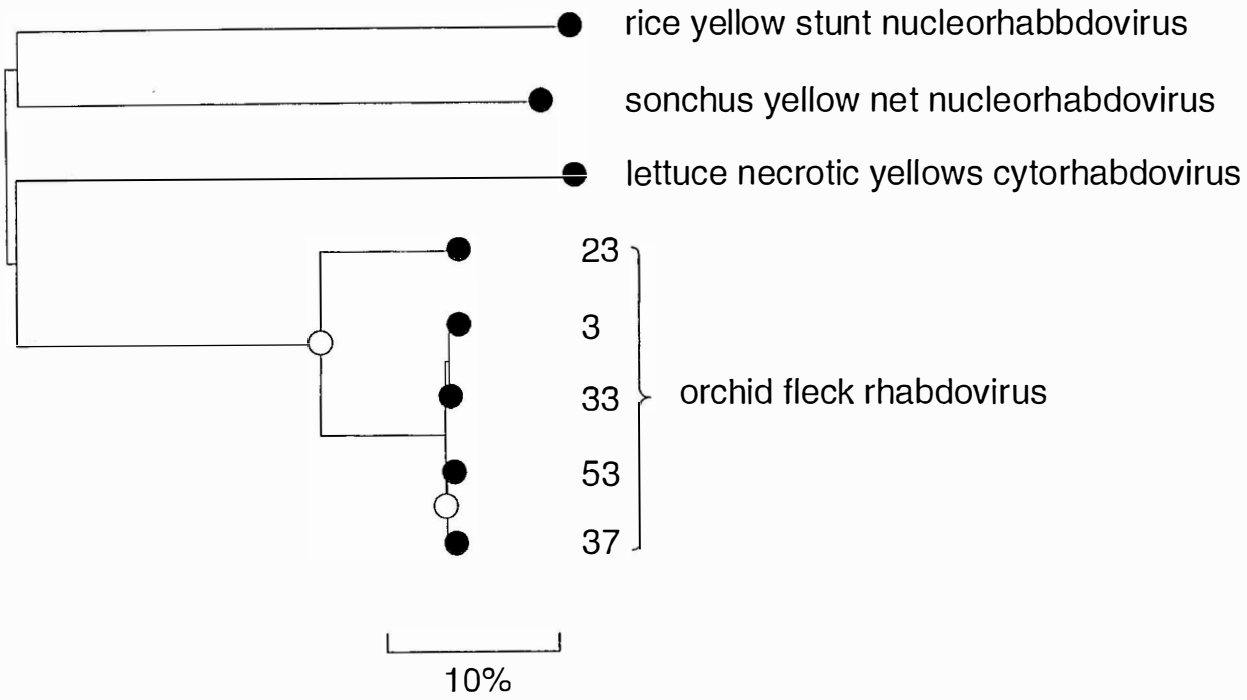
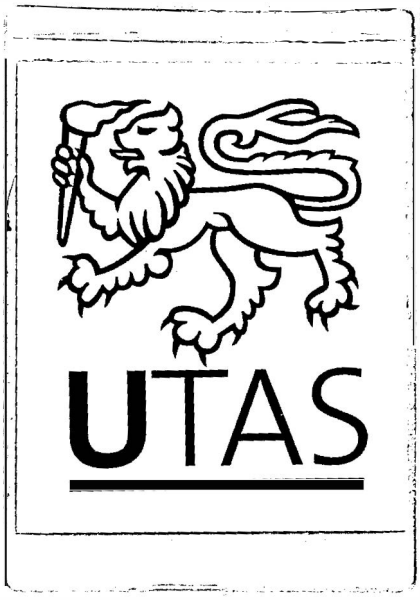


Figure 9 A dendogram calculated from the % differences between the aligned nucleotide sequences of 5 OFV isolates encoding the nucleoprotein and three rhabdovirus sequences published on the international nucleotide database.



9. Discussion

Doi *et al.* (1977) reported that sap-inoculation of OFV to alternate host plants was difficult, but successful in temperatures higher than 30°C. In this study sap inoculation was also found to be difficult and affected by glasshouse temperatures, however, very successful transmission was achieved at temperatures between 20°C and 30°C, lower than previously reported. The host range of a Tasmanian isolate of OFV, 494, was also found to be different to that previously reported. Doi *et al.* (1977) states *Nicotiana tabacum* is a suitable assay species, however *N. tabacum* never succumbed to infection in these challenge experiments suggesting it to be a non-susceptible host species to isolate 494. Kondo *et al.* (1995) also reported *N. tabacum* to be a non-susceptible host species in their study with a Japanese OFV isolate. However, contrary to the report of Kondo *et al.* (1995) *Tetragonia expansia* and *Petunia hybrida,* described as local susceptible host species for the Japanese isolate, were also found to be non-susceptible host species with isolate 494. The systemic, chlorotic lesions induced by OFV on *Chenopodium quinoa* and *C. murale* and the chlorotic, local lesions induced by OFV on inoculated leaves of *C. amaranticolor* and *Nicotiana glutinosa* in these challenge experiments were similar to those reported by Kondo *et al.* (1995).

Once inoculated to an alternate host plant, OFV could be propagated by mechanical sap inoculation, which provided an opportunity to investigate purification of the virus in hosts other than orchid. However, the extremely fragile nature of the OFV virions made purification of the virus particles difficult and this proved to be a more difficult problem to overcome than the problem of polysaccharides present in the orchid tissue. The virions could not be successfully separated from host plant contaminants because the particles degraded readily, eliminating the possibility of direct production of antibodies to be used in an immunoassay.

The production of a cDNA library of viral RNA was one way to overcome the problem of producing antibodies, however the poor quality of the purified virus preparations made the extraction of good quality viral RNA impossible. The production of a cDNA library

was not successful because high quality and suitable concentrations of viral RNA was not obtained through the methods of purifying OFV virions that were used, followed by extracting RNA from the purified preparations.

The extraction of nucleic acids from orchid leaf material for use in RT-PCR was made difficult by the presence of polysaccharides in the plant material. The spin columns of the RNeasy Plant Mini kit (QIAGEN) were blocked by the polysaccharides in the leaf material. However, the use of a wash buffer step in the total nucleic acid extraction method of Mackenzie *et al.* (1998) overcame this problem and allowed routine detection of OFV from all but seven air dried samples.

The absence of a PCR product with the mN2 primer used on some of the dried samples, although they were known to be positive for OFV, is probably due to the breakdown of viral RNA while the leaves dried and was not unexpected. Freezing or drying the leaves over calcium chloride were more suitable methods for long term storage.

Our RT-PCR tests with primers targeting a 3' terminal region of the OFV nucleoprotein genome have confirmed that one viral species, OFV, was present in all of the orchid samples we tested that showed orchid fleck virus symptoms and that contained OFV-like bacilliform virions. RT-PCR tests confirmed the enveloped bacilliform particles distinctly larger than OFV contained in the sample from Hawaii were not OFV. The sequences of the DNA fragments produced by RT-PCR from the OFV infected plants have shown that there are at least two strains of OFV; one was found in a single plant from Germany, 33 isolates of the other were found in orchids from four different continents.

OFV isolate 494 showed 82.4% nucleotide identity to the nucleoprotein gene of the Japanese isolate cloned and used to design the PCR primers mN2 and mP1. Partial amino acid sequences of the nucleoprotein of the Australian isolate showed 97.0% identity to the Japanese isolate (7 amino acid changes). The absence of a PCR product

with the phosphoprotein primer may be explained by differences between the mP1 primer and the viral sequences of the Australian, Brazillian and German OFV isolates. The mP1 primer is located immediately upstream of the phosphoprotein ORF of the Japanese isolate, therefore the 5' untranslated region of the phosphoprotein gene of OFV is probably variable. The significant diversion (>10% nucleotide divergence) of all OFV isolates tested in this study from the Japanese isolate from which the primer sequences were derived, and the failure of the primer to part of the phosphoprotein gene to produce a PCR product for any tested isolate is strong evidence that the Japanese OFV isolate may represent a third distinct strain of OFV or a different virus species although more sequence data is required to confirm this.

In searches of the international gene sequence databases, the OFV sequences only matched significantly with the sequences of other plant rhabdoviruses. However comparing these sequences did not clarify relationships among the Rhabdoviridae as, in the targeted region, the two nucleorhabdovirus sequences were no closer to one another than either was to the single cytorhabdovirus sequence or the OFV sequences. This, along with the report that OFV has a split genome (Kondo *et al.*, 1998) indicates that OFV should not be considered a member of the plant rhabdovirus family. However, it is unlikely that the relationships of rhabdoviruses will be clarified until more gene sequence data has been obtained.

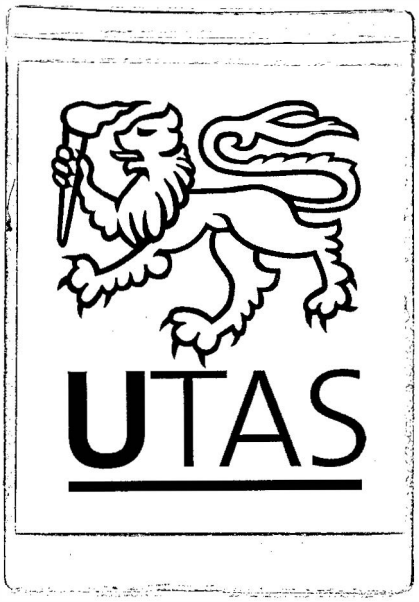
That the native orchid, *Dendrobium kingianum*, was infected by OFV in the wild suggests OFV is endemic in Australian orchid flora or transmission to wild orchids has occurred. OFV transmission by a mite, *Brevipalpus californicus*, has been demonstrated (Maedai et al., 1998) and could facilitate natural spread of the virus, although studies to determine the presence of this mite in the wild have not been conducted.

The results obtained using RT-PCR and the mN2 primer confirm that the small rhabdovirus-like particles infecting orchids around Australia, Brazil and Germany are one and the same virus, orchid fleck nucleorhabdovirus and is probably the same as the

viruses reported by Doi *et al.* (1969), Chang *et al.* (1973), Petzold (1971), Duvel and Peters (1971), Lesemann and Begtrup (1971), Begtrup (1972), Lesemann and Doraiswamy (1975) and Kitajima *et al.* (1974), and possibly that reported by Kondo *et al.* (1995). The sequences of the isolates of OFV suggest that there are small but significant isolate differences in OFV. They also show that the PCR system is extremely sensitive and reliably detects and identifies three distinct strains of OFV or possibly two strains and a second distinct by related virus species. The results obtained using RT-PCR and the mN2 primer also suggest the large rhabdovirus-like particle detected in the orchid sample from Hawaii is distinct from OFV and is probably the same virus reported by Ali *et al.* (1974), Lawson and Ali (1975) and Peters (1977). Virions of rhabdoviruses are easily altered *in vitro* by negative-staining procedures (Jackson *et al.*, 1987). Even when the fragile virions are stabilized with fixatives prior to negative staining, they may still break or swell. These problems undoubtedly contribute to the variation in size estimates of bacilliform particles observed in orchids in different laboratories. Even though the differences in lengths and widths of OFV-like particles reported in the literature could be considered sufficiently distinct, the results obtained using RT-PCR suggest they are the one virus. Variation in preparative procedures for electron microscopy can contribute considerably to the relative degree of flattening, shrinking or swelling of the fragile particles (Jackson et al., 1987). The particles may also become distorted or may even fragment during fixing and staining. Also the accuracy of measurements obtained in different laboratories is difficult to evaluate.

The failure to obtain an amplified product from one isolate of CoRSV, citrus leprosis virus or the schefflera, hibiscus, ivy and ligustrum leaves using the OFV primers suggests citrus leprosis, ligustrum ringspot, and the ringspots on schefflera, hibiscus and ivy are caused by viruses different to OFV. Electron microscopy studies by Kitajima, E.W. and Chagas, C.M. (pers. comm.) show that CoRSV samples often contain two distinct bacilliform virions. This has also been shown for citrus leprosis virus (Colariccio *et al.*, 1995). The positive RT-PCR results with one of the CoRSV samples suggests the sample may contain both OFV and CoRSV. The other sample may contain only CoRSV, which

is possibly a different virus to OFV. OFV has been shown to be transmitted by the mite *Brevipalpus californicus* (Maeda et al., 1998). Mite vectors in the genus *Brevipalpus* have also been demonstrated for CoRSV, citrus leprosis virus, ligustrum ringspot and green spot on passionfruit (Colariccio *et al.*, 1995, Rossetti *et al.*, 1998), suggesting the viruses may be related. However, a study of the relationship between these viruses needs to be repeated with a higher sample number. RT-PCR primers are currently being designed for CoRSV (Nogueira *et al.*, pers. comm.) and further tests incorporating these primers will yield conclusive results. RT-PCR tests showing a common violet contained an isolate of the commonest OFV strain need further investigation and may show that OFV has a wider host range than originally thought.



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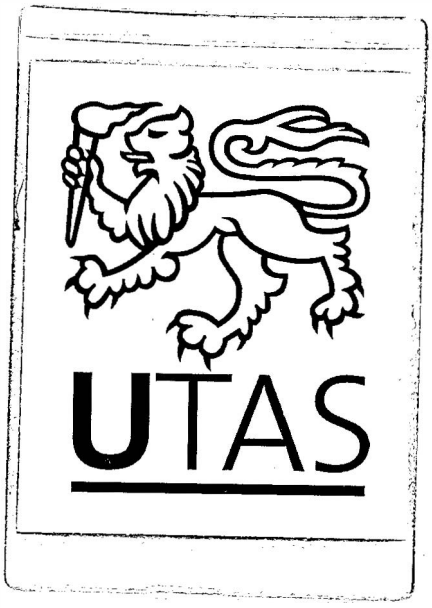


11. Appendices

11.1 Appendix 1

Mechanical inoculation buffers

0.01M phosphate buffer, pH 7.2

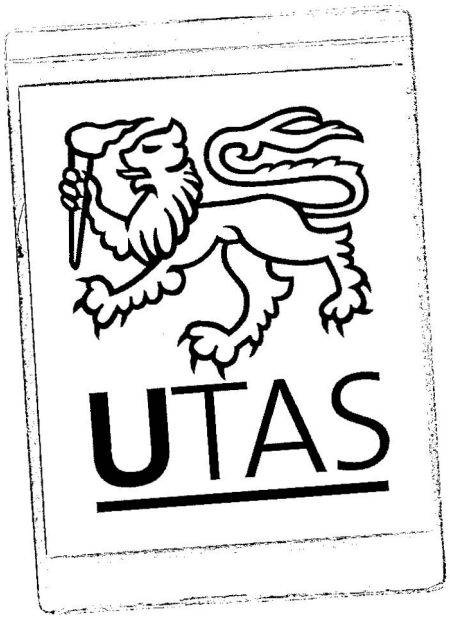
7.17ml 1M di-potassium hydrogen orthophosphate
2.83ml 1M potassium di-hydrogen orthophosphate

Dilute combined 1M stock solution to 1000ml with distilled water.
Adjust to pH 7.2 if necessary.

0.02M HEPES buffer, pH 7.4 (Lesemann, D-E, per. comm.)

0.02M N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
2% polyvinyl-pyrrolidone (MW 40,000)
0.2% sodium sulphite

Adjust to pH 7.4



Virion purification buffers

OFV Extraction buffer (Chang *et al.* 1976)

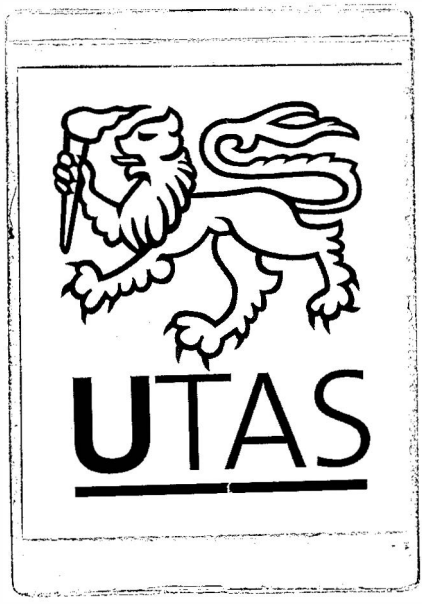
0.1M phosphate buffer, pH 7.0
0.1M sodium diethyldithiocarbamate
0.1% L-ascorbic acid
5% Triton X-100
0.5% sodium deoxycholate

SCV Extraction buffer (Hunter *et al.* 1990)

100mM Tris-HCl, pH 8.2
10mM magnesium acetate
40mM sodium sulfite
1mM manganese chloride

SYNV Extraction buffer (Jackson & Christie, 1977)

0.1M Tris-HCl, pH 8.4
0.01M magnesium acetate
0.04M sodium sulfite
0.001M manganese chloride



Luria Broth (LB)

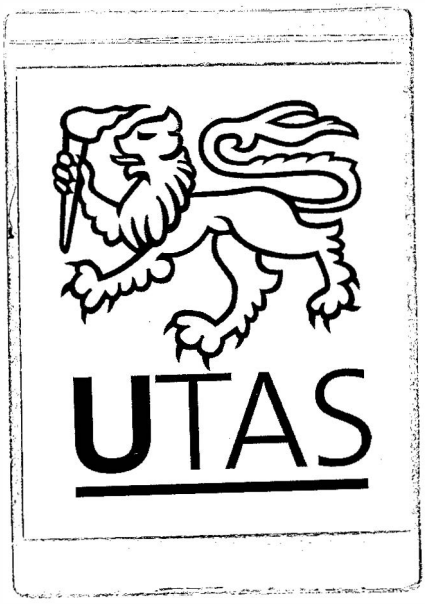
LB liquid, for 1 litre

10g NaCl
10g tryptone
5g yeast extract

Adjust to pH 7.0

LB agar, for 1 litre
10g NaCl
10g tryptone
5g yeast extract

Adjust to pH 7.0
Add 20g agar



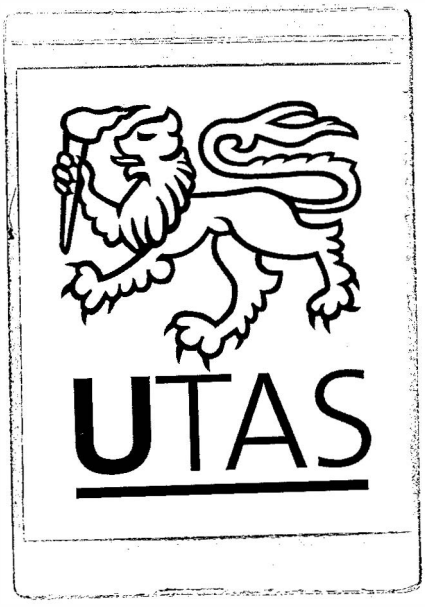
Small Scale Preparations of Plasmid DNA (Sambrook *et al.*, 1989)

Solution I
50 mM glucose
25 mM Tris.Cl (pH 8.0)
10 mM EDTA (pH 8.0)

Solution II
0.2 N NaOH
1% SDS

Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml



Nucleic acid extraction

CTAB buffer (Campbell, P. pers comm.)

2g CTAB (cetyl trimethyl ammonium bromide)
20 ml 0.5 M Tris-HCl, pH8.0
4 ml 0.5 M EDTA,pH8.0
28 ml 5M NaCl
1g polyvinyl-pyrrolidone (MW 40,000)
47 ml water

Wash buffer (Mackenzie *et al.*, 1998)

0.1g bovine serum albumin
80 ml 5M NaCl
0.4 ml 0.5 M EDTA, pH8.0
2 ml 1M Tris-HCl, pH8.0

Make up to 200 ml with distilled water

CTAB buffer (Mackenzie *et al.*, 1998)

2g CTAB (cetyl trimethyl ammonium bromide)
10 ml 1M Tris-HCl, pH8.0
28 ml 5M NaCl
500 µl β-mercaptoethanol

Make up to 100ml with distilled water.

STE buffer (Braithwaite *et al.*, 1995)

0.1M NaCl
10mM Tris-HCl, pH 8.0
1mM EDTA, pH 8.0

11.2 Appendix 2

CLUSTAL V multiple sequence alignment of OFV isolates

```
ALB003          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
ALB005          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB007          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
ALB009          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB011          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
ALB013          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
ALB017          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB019          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB023          TGTGCCATAGCAGATGAGACGCACCACACCAATAGGAAGAGGCACGGACT
ALB025          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB027          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB029          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB031          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
ALB033          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
ALB037          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB039          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB041          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB043          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB045          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB047          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
                ******** *********** *********** *********** ** **

ALB003          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACGGGAATGATTCCGT
ALB005          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB007          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACAGGAATGATTCCGT
ALB009          TCTCATGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB011          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACGGGAATGATTCCGT
ALB013          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACAGGAATGATTCCGT
ALB017          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB019          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB023          TCTCAGATTCCTAATCCTTCAACATGTGGATCTCACGGGGATGATCCCAT
ALB025          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB027          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB029          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB031          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACGGGAATGATTCCGT
ALB033          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACGGGAATGATTCCGT
ALB037          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB039          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB041          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB043          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB045          TCTCAGGTTCCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB047          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
                ***** ** ** ******** ********** ** ** ***** ** *

ALB003          ATGGAATGTATATCGATATGAGGAGACACTTCACCCTATTGACACCAGGT
ALB005          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB007          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB009          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB011          ATGGAATGTATATCGATATGAGGAGACACTTCACCCTATTGACACCAGGT
ALB013          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB017          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB019          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB023          ATGGAATGTACATTGACATGCGAAGGCACTTCACCCTCTTAACACCCGGC
ALB025          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB027          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
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ALB029          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB031          ATGGAATGTATATCGATATGAGGAGACACTTCACCCTATTGACACCAGGT
ALB033          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB037          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB039          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB041          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB043          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB045          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB047          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
                ********** ** ** *** * ** ********** ** ***** **

ALB003          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
ALB005          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCGGTGT
ALB007          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
ALB009          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
ALB011          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
ALB013          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
ALB017          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
ALB019          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCGGTGT
ALB023          CAGTTGCTAACATGGCTTCATGACAACCAGGTATCTAGGCCTCTCAGTGT
ALB025          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCGGTGT
ALB027          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCGGTGT
ALB029          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCGGTGT
ALB031          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
ALB033          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
ALB037          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCGGTGT
ALB039          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCGGTGT
ALB041          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
ALB043          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCGGTGT
ALB045          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGCGT
ALB047          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
                *** * ** ******** ********** ** ** ***** *** * **

ALB003          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB005          CATAGCCGACATAAACACCAGGTATGATGTATCCAATGGGGCAGACAGGT
ALB007          CATAGCCGACATAAACACCAGGTATGATGTGTCTAATGGGGCAGACAGGT
ALB009          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB011          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB013          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB017          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB019          CATAGCCGACATAAACACCAGGTATGATGTATCCAATGGGGCAGACAGGT
ALB023          GATTGCGGACATAAACACCAGGTATGATGTTTCTAACGGGTCAGATCGGT
ALB025          CATAGCCGACATAAACACCAGGTATGATGTATCCAATGGGGCAGACAGGT
ALB027          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB029          CATAGCCGACATAAACACCAGGTATGATGTATCCAATGGGGCAGACAGGT
ALB031          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB033          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB037          CATAGCCGACATAAACACCAGGTATGATGTATCCAATGGGGCAGACAGGT
ALB039          CATAGCCGACATAAACACCAGGTATGATGTATCCAATGGGGCAGACAGGT
ALB041          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB043          CATAGCCGACATAAACACCAGGTATGATGTATCCAATGGGGCAGACAGGT
ALB045          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB047          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
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ALB003          TCTGGAGATACTCAAGGGGGCTAGACCCAGGGTTCTTTATAGCTCTGCAA
ALB005          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB007          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB009          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB011          TCTGGAGATACTCAAGGGGGCTAGACCCAGGGTTCTTTATAGCTCTGCAA
ALB013          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
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ALB017          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB019          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB023          TCTGGAGGTACTCAAGGGGTCTAGACCCAGGATTCTTCATAGCTTTACAA
ALB025          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB027          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB029          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB031          TCTGGAGATACTCAAGGGGGCTAGACCCAGGGTTCTTTATAGCTCTGCAA
ALB033          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB037          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB039          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB041          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB043          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB045          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB047          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
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ALB003          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB005          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB007          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB009          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB011          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB013          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB017          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB019          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB023          CAGTCAAAATGTGTCACTCTGATAGCCAGGATGGCACATATACTGGTGAA
ALB025          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB027          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB029          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB031          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB033          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB037          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB039          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB041          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB043          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB045          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB047          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
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ALB003          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB005          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB007          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB009          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB011          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB013          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB017          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB019          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB023          GGGAGGAGCCGTCGCTGTCAATGAATACTCCGATCCTCGAAAGGCAAAGT
ALB025          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB027          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB029          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB031          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB033          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB037          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB039          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB041          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB043          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB045          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB047          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
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ALB003          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
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ALB005          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB007          CATTGGAGAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB009          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB011          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB013          CATTGGAAAACAAACCCGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB017          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB019          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB023          CATTGGAGAACAAGCCTGGGCTGGCAGCGGAGGCAGACAAGTTCGCCACT
ALB025          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB027          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB029          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB031          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB033          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB037          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB039          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB041          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB043          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB045          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB047          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
                ******* ***** ** ** ** ** ******** ******** *****

ALB003          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB005          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB007          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB009          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB011          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB013          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB017          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB019          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB023          GAGTTCGTGGAGGCATACAACGGCCTATCAGGATCAAGTGCAAACGCCGG
ALB025          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB027          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB029          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB031          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB033          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB037          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB039          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB041          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB043          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB045          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB047          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
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ALB003          TCCTGTATCTCGCAAGCTCTACAACCAAGGCAGAGGCATCCCAACTCGCA
ALB005          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB007          TCCTGTATCTCGCAAGCTCTACAATCAGGGCAGAGGCATCCCAACTCGCA
ALB009          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB011          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
ALB013          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
ALB017          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB019          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB023          CCCTGTGTCCCGCAAACTCTACAACCAGGGCAGAGGCATCCCGACCCGCA
ALB025          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB027          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB029          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB031          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
ALB033          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
ALB037          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB039          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB041          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
ALB043          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
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ALB045          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB047          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
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ALB003          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB005          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB007          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB009          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB011          GAGGTCTGTTCACACCGCCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB013          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB017          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB019          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB023          GGGGCCTGTTCACACCCCCTTCCGCCAGACCCGCGCCCGTTGTGAATGTA
ALB025          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB027          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB029          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB031          GAGGTCTGTTCACACCGCCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB033          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB037          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB039          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB041          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB043          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB045          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB047          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
                * ** *********** ** *************** ** ** ***** **

ALB003          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAG
ALB005          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB007          CATGTTCCAGCCCCGTCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB009          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB011          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB013          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB017          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB019          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB023          CACGTCCCTGCCGCCTCGTCCTCTCTGGCAGGGGCCCTGGATGCCATGAA
ALB025          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB027          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB029          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB031          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB033          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB037          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB039          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB041          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB043          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB045          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB047          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCCCTGGATGCCCTGAA
                ** ** ** *** * ** ** ** ******** ** ********* ***

ALB003          CAATGAATAA
ALB005          CAATGAATAA
ALB007          CAATGAATAA
ALB009          CAATGAATAA
ALB011          CAATGAATAA
ALB013          CAATGAATAA
ALB017          CAATGAATAA
ALB019          CAATGAATAA
ALB023          CAGTGACTGA
ALB025          CAATGAATAA
ALB027          CAATGAATAA
ALB029          CAATGAATAA
ALB031          CAATGAATAA
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ALB033          CAATGAATAA
ALB037          CAATGAATAA
ALB039          CAATGAATAA
ALB041          CAATGAATAA
ALB043          CAATGAATAA
ALB045          CAATGAATAA
ALB047          CAATGAATAA
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CLUSTAL V multiple sequence alignment of OFV isolates

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ALB039          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB041          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB043          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB045          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB047          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB052          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB053          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
ALB056          TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCACGGTCT
OR237           TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
OR494           TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
OR590           TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
OR591           TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
OR593           TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
AM773           TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
AM775           TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
AM776           TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
AM777           TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
AM778           TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
AM780           TGTGCCATTGCAGATGAGACTCACCACACCAACAGGAAGAGGCATGGTCT
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ALB039          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB041          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB043          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB045          TCTCAGGTTCCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB047          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB052          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
ALB053          TCTCAGGTTCCTGATCCTTCAGCATGTGGATCTAACAGGAATGATTCCGT
ALB056          TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTGACAGGAATGATTCCGT
OR237           TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACGGGAATGATTCCGT
OR494           TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACGGGAATGATTCCGT
OR590           TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACAGGAATGATTCCGT
OR591           TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACAGGAATGATTCCGT
OR593           TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACAGGAATGATTCCGT
AM773           TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACGGGAATGATTCCGT
AM775           TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACGGGAATGATTCCGT
AM776           TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACGGGAATGATTCCGT
AM777           TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACGGGAATGATTCCGT
AM778           TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACGGGAATGATTCCGT
AM780           TCTCAGGTTTCTGATCCTTCAGCATGTGGATCTAACGGGAATGATTCCGT
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ALB039          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB041          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB043          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB045          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB047          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB052          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
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ALB053          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
ALB056          ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
OR237           ATGGAATGTATATCGATATGAGGAGACACTTCACCCTATTGACACCAGGT
OR494           ATGGAATGTATATCGATATGAGGAGACACTTCACCCTATTGACACCAGGT
OR590           ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
OR591           ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
OR593           ATGGAATGTATATCGATATGCGAAGACACTTCACCCTATTGACACCAGGT
AM773           ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCCGGT
AM775           ATGGAATGTATATCGATATGAGGAGACACTTCACCCTATTGACACCAGGT
AM776           ATGGAATGTATATCGATATGAGGAGACACTTCACCCTATTGACACCAGGT
AM777           ATGGAATGTACATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
AM778           ATGGAATGTATATCGATATGAGAAGACACTTCACCCTATTGACACCAGGT
AM780           ATGGAATGTATATCGATATGAGGAGACACTTCACCCTATTGACACCAGGT
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ALB039          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCGGTGT
ALB041          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
ALB043          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCGGTGT
ALB045          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGCGT
ALB047          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
ALB052          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
ALB053          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGCGT
ALB056          CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
OR237           CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
OR494           CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
OR590           CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCCGGCCCCTCAGTGT
OR591           CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCCGGCCCCTCAGTGT
OR593           CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
AM773           CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
AM775           CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
AM776           CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGCGT
AM777           CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCTCTCAGTGT
AM778           CAGCTACTGACATGGCTCCATGACAACCAAGTCTCCAGGCCCCTCAGTGT
AM780           CAGCTACTGACATGGCTCCATGATAACCAAGTCTCCAGGCCCCTCAGCGT
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ALB039          CATAGCCGACATAAACACCAGGTATGATGTATCCAATGGGGCAGACAGGT
ALB041          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB043          CATAGCCGACATAAACACCAGGTATGATGTATCCAATGGGGCAGACAGGT
ALB045          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB047          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB052          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
ALB053          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGA
ALB056          CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
OR237           CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
OR494           CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
OR590           CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
OR591           CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
OR593           CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
AM773           CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
AM775           CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
AM776           CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
AM777           CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
AM778           CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
AM780           CATAGCCGACATAAACACCAGGTATGATGTGTCCAATGGGGCAGACAGGT
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ALB039          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB041          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB043          TCTGGAGATACTCAAGGGGGCTTGACCC AGGGTTCTTTATAGCTCTGCAA
ALB045          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
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ALB047          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB052          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB053          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
ALB056          TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
OR237           TCTGGAGATACTCAAGGGGGCTAGACCCAGGGTTCTTTATAGCTCTGCAA
OR494           TCTGGAGATACTCAAGGGGGCTAGACCCAGGGTTCTTTATAGCTCTGCAA
OR590           TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
OR591           TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
OR593           TCTGGAGATACTCAAGGGGGCTTGACCCGGGGTTCTTTATAGCTCTGCAA
AM773           TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
AM775           TCTGGAGATACTCAAGGGGGCTAGACCCAGGGTTCTTTATAGCTCTGCAA
AM776           TCTGGAGATACTCAAGGGGGCTAGACCCAGGGTTCTTTATAGCTCTGCAA
AM777           TCTGGAGATACTCAAGGGGGCTAGACCCAGGGTTCTTTATAGCTCTGCAA
AM778           TCTGGAGATACTCAAGGGGGCTTGACCCAGGGTTCTTTATAGCTCTGCAA
AM780           TCTGGAGATACTCAAGGGGGCTAGACCCAGGGTTCTTTATAGCTCTGCAA
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ALB039          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB041          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB043          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB045          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB047          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB052          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB053          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
ALB056          CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
OR237           CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
OR494           CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
OR590           CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
OR591           CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
OR593           CAGTCAAAGTGCGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
AM773           CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
AM775           CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
AM776           CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
AM777           CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
AM778           CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
AM780           CAGTCAAAGTGTGTCACTCTAATAGCCAGGATGGCTCACATATTGGTGAA
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ALB039          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB041          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB043          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB045          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB047          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB052          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB053          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
ALB056          GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
OR237           GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
OR494           GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
OR590           GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
OR591           GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
OR593           GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
AM773           GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
AM775           GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
AM776           GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
AM777           GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
AM778           GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
AM780           GGGTGGAGCTGTAGCCGTCAATGAATACTCTGATCCTAGGAAGGCAAAGT
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ALB039          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB041          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
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ALB043          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB045          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB047          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB052          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB053          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
ALB056          CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
OR237           CATTGGAAAACAAGCCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
OR494           CATTGGAGAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
OR590           CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
OR591           CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
OR593           CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
AM773           CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
AM775           CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
AM776           CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
AM777           CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
AM778           CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
AM780           CATTGGAAAACAAACCTGGACTTGCTGCGGAGGCTGACAAGTTTGCCACA
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ALB039          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB041          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB043          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB045          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB047          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB052          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB053          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
ALB056          GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
OR237           GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
OR494           GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
OR590           GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
OR591           GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
OR593           GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
AM773           GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
AM775           GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
AM776           GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
AM777           GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
AM778           GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
AM780           GAGTTTGTGGAGGCATACAATGGCCTGTCCGGATCAAGCGCCAATGCAGG
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ALB039          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB041          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
ALB043          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB045          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB047          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
ALB052          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
ALB053          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
ALB056          TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACCCGCA
OR237           TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
OR494           TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
OR590           TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
OR591           TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
OR593           TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
AM773           TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
AM775           TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
AM776           TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
AM777           TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
AM778           TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
AM780           TCCTGTATCTCGCAAGCTCTACAATCAAGGCAGAGGCATCCCAACTCGCA
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ALB039          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB041          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB043          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB045          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB047          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB052          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB053          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
ALB056          GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
OR237           GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
OR494           GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
OR590           GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
OR591           GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
OR593           GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
AM773           GAGGTCTGTTCACACCACCATCCGCCAGACCTGCACCTGTCGTGAACGTG
AM775           GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
AM776           GAGGTCTGTTCACACCGCCATCCGCCAGACCCGCACCTGTCGTGAACGTG
AM777           GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
AM778           GAGGTCTGTTCACACCACCATCCGCCAGACCCGCACCTGTCGTGAACGTG
AM780           GAGGTCTGTTCACACCGCCATCCGCCAGACCCGCACCTGTCGTGAACGTG
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ALB039          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB041          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB043          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB045          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB047          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCCCTGGATGCCCTGAA
ALB052          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB053          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
ALB056          CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
OR237           CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
OR494           CATGTTCCAACCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
OR590           CATGCTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
OR591           CATGCTCCAACCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
OR593           CATGTTCCAACCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
AM773           CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
AM775           CATGTTCCAACCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
AM776           CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
AM777           CATGTCCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
AM778           CATGTTCCAGCCCCATCATCATCCTTGGCAGGAGCACTGGATGCCATGAA
AM780           CATGTTCCAGCCCCATCATCATCCCTGGCAGGAGCACTGGATGCCATGAA
                **** *** ********** ********** ********* ****

ALB039          CAATGAATAA
ALB041          CAATGAATAA
ALB043          CAATGAATAA
ALB045          CAATGAATAA
ALB047          CAATGAATAA
ALB052          CAATGAATAA
ALB053          CAATGAATAA
ALB056          CAATGAATAA
OR237           TAA-XXX-A-
OR494           CAATGAATAA
OR590           CAATGAATAA
OR591           CAATGAATAA
OR593           CAATGAATAA
AM773           CAATGAATAA
AM775           CAATGAATAA
AM776           CAATGAATAA
AM777           CAATGAATAA
AM778           CAATGAATAA
AM780           CAATGAATAA
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Clustal W multiple sequence alignment of OFV, RYSV, SYNV and LNYV.

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        1            15 16           30 31           45 46           60 61           75 76           90
1 RYSV ACATATCTGAC-ACT GCGAGCC----ACAG TTGCTTAT--AATAT AGCCAGTGCAGATGC --CTTATTAGTCCCT GAAA-ATGTCAATTA     80
2 OFV  -----TGTGATGACC TCAAGGCT---ACTC TTTGCCAT--CACTG TGCCATTGCAGATGA GACTCACCACACCAA CAGG-AAG--AGGCA     77
3 SYNV -------ACATTCCC TAGAGTCAAGAACAC TTTGATATTGCATGT TGCACATGCAGA-GA CATACTTCAGACCTA CTCCCAAGATCTTCA     82
4 LNYV ---------------- ---------------- ---------------- -------GCAGT-GA ATGAGAACAGAACCC CTGGATTGCTAGTGA     37

        91          105 106          120 121          135 136          150 151          165 166          180
1 RYSV TGGCCTGTGCAGGAT GCTTGTGTTCCAGCA CCTAGAACTTAGTGG CCTTCAACTTTATAA AATGGCCA-TGACCC TCATTGCTCATTTCA    169
2 OFV  TGGTCTTCTCAGGTT TCTGATCCTTCAGCA TGTGGATCTAACGGG AATGATTCCGTATGG AATGTATA-TCGATA TGAGGAGACACTTCA    166
3 SYNV ATGTC--CTCAGGTT CTTGTTCTTTCAGAA TCTAGAATTTATGGG ATTACATGCATATGT GA-GCATAGTCACAA TCATGTCAAAGGTCG    169
4 LNYV --CTCAGCAAGGGTT ACTGAA-TTACCTTG CCTGCCAACAATTTG CCTACCCTGGTATGC -ATGCATA--CACTC TCTTGATAGAGAT-A    120

        181          195 196          210 211          225 226          240 241          255 256          270
1 RYSV AACTGATAGAG-CCA AATAAATTTCTCTCA TGGATTTATGACCCT C---TCTCTGAGGCG TCTATAGATCAGATC T---AT-------AA    245
2 OFV  CCCT-ATTGACACCA GGTCAGCTACTGACA TGGCTCCATGACAAC CAAGTCTCC-AGGCC CCTCAGTGTCATAGC CGACAT-------AA    247
3 SYNV CACT---C-CCTCCT AGTCAAGTACTCTCA TGGTTGAGGGTCAGT G---GGTCAGAGATG GCAATTGATGAG-GC TTTCATGATCATGAA    251
4 LNYV CACGAGCACACGGGT A-TGAAGTTTAGTGA TTTGCTAGTGGAGAT GGATTGTCC--GGCC ACTAGAGCAGGTGTA CGGGAGGCTCTGGAG    207

        271          285 286          300 301          315 316          330 331          345 346          360
1 RYSV G--ATAGCTGTTAAT T--ATGA----CAAT GTGAACTCCAAAACC CACAAACATTGGAAG TATGCCAAACTTG-- ---CCAGGGGACAAT    322
2 OFV  ---ACACCAGGTA-T G--ATGTGTC-CAAT GGGGCAGACAGGTTC TGGAGATACTC-AAG GGGGCTAGAC----- ---CCAGGGTTCTTT    321
3 SYNV C--ACTCTTGATAAT GGAATGATTGACAAT G-G--CCATAATGCC GAGAGATTGTGGAAG TACGCTCGATGTTTA GATCAAGGGTATTTT    336
4 LNYV TTGATAAGAGATTAT GAGATAAC---CAAA GATCATCCTAAAAGA ACCACTTACTTTACA TATGCTAGGAATT-- ------GGGATCCAA    286

        361          375 376          390 391          405 406          420 421          435 436          450
1 RYSV ATTGG-CTG-AACAC T---ACTGTGAAG-- --AGGAACCAGTTTT TGGCTTACATTCTTG CAGATCTCGAGCTAA AATACGGCCTAGCTG    403
2 OFV  ATAGCTCTGCAACAG TCAAAGTGTGTCACT CTAATAGCCAGGAT- -GGCTCACATA-TTG GTGAA---GGGTGGA GCTG-----TAGCCG    400
3 SYNV AACAGGCTTCAATCA TCCTATTCAGCAGAA TTGATTGCTATGTT- -AGCATACATT---- --GAA-----ATAAA TATGGGAATTAGCAC    413
4 LNYV AATATTTTGGGGCAC TAC-AATCTACAG-- --AGT-GCAAGACTC TCGTATACGTTGCTG C-GTCTGTCAGTAAG AAGG-----TCTCTG    364

        451          465 466          480 481          495 496          510 511          525 526          540
1 RYSV GGAAATCTGA-CTAC TCCAGTCCCAAAAGA ATG-AAAGCCTTAAG TGG-GATGCCGGTT- --GAACGAATGACAG AGGCAGAGACCATCT    487
2 OFV  TCAA---TGA-ATAC TCTGATCCTAG--GA AGGCAAAG---TCAT TGGAGAACAAACCT- --GGACTTGCTGCGG AGGCTGACAAGTTTG    478
3 SYNV GGAAGTGGGATATAA T----TCACCGTTAA ATATATATGC--CAT AGC-GAACAA---T- --AAAGCAGTAAAGG AGGTTG-GAAGAATG    489
4 LNYV CTCAGGGCGC-AAAC G---GTGACCCT--A -TGGAAAT-----AT TTGCCATCAAAAATC TCGACGCAACCATCA AGGCTC---GGTTAG    439

        541          555 556          570 571          585 586          600 601          615 616          630
1 RYSV CCAAGGCGGTAGAGC AAATGTACACCGCAA TAGAGAGTGCAAAGA GAGTTGATGCAGGAG CTGCTTATAGATTGG CGAAGAAACTCGGCC    577
2 OFV  CCACAGAGTTTGTGG AGGCATACAATGGCC T---GTCCGGATCAA GCGCCAATGCAGGTC CTGTATCTCGCAAGC TCTACAATCAAGG--    563
3 SYNV --AAAGCAGATGTGT --TCATACAGTGCAA AA-ACTCCGTGGTTT -CTTTGACCCAGGA- -TGCTTCCGTCATAG ACAAGG-TCTATGCT    570
4 LNYV ACCCCGTGGCCGAGA --ATATGGCAGGTAA GA--TATTGGATCAA ATGCTTATGGATGAG ATGTCTGGAGCATCG TGGGCGACAAAGG--    523
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      631         645 646         660 661         675 676         690 691         705 706         720
1 RYSV CACCCAGAGCGAACG CACACAGCAGGCGCA AGGAGCC-CAACAAC AGCAGG-CAGCACAG GGACAAGCAGCCCAA CAGCAAGCAGCAGGG    665
2 OFV  ----CAGAGGCATCC CAACTCGCAGAGGTC TGTT-CA-CACCACC ATCCGC-CAG----- --ACCCGCA-CCTGT CGTGAAC-----GTG    633
3 SYNV GCAGCACAACAAAAA CACATCAGATCAG-A AGAAGCTGCACGACC ATCTGAACAGA---- --ACAAGGAGGATGA GGTGGTT--GCAATG    651
4 LNYV ----CAT--C-AACA CAATGAAGAGTTG-A TGT--CATTA--AAC ATTTATGCGTATTTG ATACTAT-A--CTTA TATCACTTGTTACTG    598

      721         735 736         750 751         765 766         780 781         795 796         810
1 RYSV CAGGGACAAGCGCAA -CAAGCATCAGGTTC TGGGACG-GC----- ACAG-CAAGCCTCAG GCTCAGGGACCGCCC AACAAGCAA-CAGGA    746
2 OFV  CATGTTCCAACCCCA -TCATCATCC----C TGGCAGGAGC----- ACTG-GATGCC--AT GAACAATGA------ -ATAA----------    693
3 SYNV GACACAGATGCCCCA TCCAGGAAAAGAAGG AGTGACGCTCTTACA ACTGAGAAGCCT-AA GAAGGCTCTCCCTGC AATAATCAAACTACC    740
4 LNYV AAGATTTAAGAAAA- ---------------- ---------------- ---------------- ---------------- ----------    612

      811         825 826         840 841         855 856         870 871         885 886         900
1 RYSV TCTGGGGCCGCACAA CAACAAGCCCAAGGA AGCCAGCAGACCCCC GCAACAGGACAGGCA GCAGCTGGCACAGCC ATGGAGGTTGACAAG    836
2 OFV  ---------------- ---------------- ---------------- ---------------- ---------------- ----------    693
3 SYNV AAACATACCGGACTT TTAATAAACTACAGC CACAACTCTACCTCC CCCATTATGAATAAA CGACCTAACATATAA TATAAG----------    821
4 LNYV ---------------- ---------------- ---------------- ---------------- ---------------- ----------    612

      901         915 916         930 931         945 946         960 961         975 976         990
1 RYSV AAGGCAGAGGGGAGC CAGAGGGCACAGGAC GCAAACGCTCTCGGG AATGTTCTGTAAAGG AACTTGCCAATGTAT CCAATAATAATGTCA    926
2 OFV  ---------------- ---------------- ---------------- ---------------- ---------------- ----------    693
3 SYNV ---------------- ---------------- ---------------- ---------------- ---------------- ----------    821
4 LNYV ---------------- ---------------- ---------------- ---------------- ---------------- ----------    612

      991        1005 1006       1020 1021       1035 1036       1050 1051       1065 1066       1080
1 RYSV TGCATTACTGTAAAG CATGGCCTGTGTGTA TTCTTAATTATATAA TATGCCTCACTTGCA GGCTGGTGTCTTGTA CTGTTCTCTATCCAC   1016
2 OFV  ---------------- ---------------- ---------------- ---------------- ---------------- ----------    693
3 SYNV ---------------- ---------------- ---------------- ---------------- ---------------- ----------    821
4 LNYV ---------------- ---------------- ---------------- ---------------- ---------------- ----------    612

      1081       1095 1096       1110 1111       1125 1126       1140 1141       1155 1156       1170
1 RYSV AAATCTATATATTGT TATGGTATTTATAAT AAAAA   1051
2 OFV  ---------------- ---------------- -----    693
3 SYNV ---------------- ---------------- -----    821
4 LNYV ---------------- ------- ------- -----    612
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